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PRINCIPAL INVESTIGATOR: Isaac Cohen, Ph.D.  
Jane N. Winter, M.D.

CONTRACTING ORGANIZATION: Northwestern University  
Evanston, Illinois 60208

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<b>13. ABSTRACT (Maximum 200 Words)</b>  The main goal of our project is to culture human hematopoietic stem cells to produce enough megakaryocytes (MK) to be transfused to patients as a supplement to the conventional stem cell transplant. The transfused megakaryocytes will generate platelets, eliminating the need for repeated platelet transfusions post-transplant. Growth factors (GF) are needed to grow and expand MK using an ex vivo expansion protocol. We planned, as described in our original proposal, to use GF developed by Searle. Searle was bought by Pharmacia. This left us without a source of the necessary GF for our clinical trial. We had to seek alternative sources for our studies. Fortunately, we were able to obtain clinical grade TPO and IL-3 through R&D and Flt3-L through Immunex, and we reported last year our successful small-scale study. At the time that we requested permission to change the source of GF for the scale-up study and the clinical trial, the grant and clinical protocol underwent re-review by the Army Human Subject Board. They requested that a second clinical protocol be written and submitted for review prior to initiating the scale-up protocol. This resulted in considerable delay. We describe in the present report the successful scale-up results. We were fortunate to have rapid review by the FDA to obtain an IND for the process and approval of the clinical protocol. These delays prevented us from adhering to our estimated timeline. Our first patient will be enrolled this week (10/8/2001). We are planning to apply for a supplementary grant period of one year to finish our clinical trial.		
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**INTRODUCTION:** As stated in our grant, the main goal of our project is to culture human hematopoietic stem cells to produce enough megakaryocytes (MK) to be transfused to patients as a supplement to the conventional stem cell transplant. The transfused megakaryocytes will generate platelets, eliminating the need for repeated platelet transfusions post-transplant. Growth factors (GF) are needed to grow and expand MK using an ex vivo expansion protocol.

We have described in our last Report the wavering commitment of Searle to supply us with GF following our successful experimental studies. This left us without a source of the necessary GF for our clinical trial. We had to seek alternative sources for our studies. Following several attempts to acquire GF from different companies (see Report Year 02), we were fortunate to obtain clinical grade TPO and IL-3 through R&D and Flt3-L through Immunex, and we reported last year our successful small-scale study. At the time that we requested permission to change the source of GF for the scale-up study and the clinical trial, the grant and clinical protocol underwent re-review by the Army Human Subject Board. They requested that a second clinical protocol be written and submitted for review prior to initiating the scale-up protocol. This resulted in considerable delay. We were fortunate to have rapid review by the FDA to obtain an IND for the process and approval of the clinical protocol. These delays prevented us from adhering to our estimated timeline. Our first patient will be enrolled this week (10/8/2001). We are planning to apply for a supplementary grant period of one year to finish our clinical trial.

We will describe in the Body of this report the successful results of the preclinical scale-up study, as well as the main elements of the FDA approval of our IND and the Chart Protocol and Component Quality Monitoring Matrix designed for each patient enrolled which were all finally approved by the Army IRB.

## **BODY:**

### **Large Scale Ex Vivo Expansion of Early and Late Megakaryocyte Progenitors Using rhTPO, IL-3, and Flt-3L.**

#### **Materials and Methods**

##### *Preparation of low density nonadherent mononuclear cells from bone marrow*

All samples were collected within the guidelines of the Northwestern University Institutional Review Board on Human Subjects. Bone marrow, obtained from the femur of hematologically normal patients having total hip arthroplasty, was collected in anticoagulant designed to prevent platelet activation and containing final concentrations of 50 U/ml preservative-free heparin (GIBCO/Life Technologies, Gaithersberg, MD), 1 mM Na<sub>2</sub>EDTA, and 0.1 mg/ml DNase I (Boehringer Mannheim, Indianapolis, IN) in 20 ml α-thioglycerol-free Iscove's modified Dulbecco's medium (IMDM, GIBCO). Marrow cells were repeatedly extracted from bone fragments with IMDM containing 0.1 mg/ml DNase I (Boehringer Mannheim) and 4 µg/ml gentamicin (Life Technologies). The extract was homogenized by passage through a 21 gauge needle to remove bone fragments. Low density mononucleated cells were isolated with the use of Ficoll-Paque (Pharmacia Corp., Piscataway, NJ) as described [Debili, 1991 #60]. Residual red cells were lysed with a NH<sub>4</sub>Cl-based lysis reagent (Ortho-Mune, Ortho Diagnostics, Raritan, NJ), and the remaining cells recovered by centrifugation for 6 min at 420xg through a 10% BSA-PBS (Sigma, St. Louis MO) cushion.

##### *Collection of Peripheral Blood Progenitor Cells*

After informed consent, patients were mobilized with rhu-G-CSF (Filgrastim, Immunex, Seattle, WA) prior to harvest, and PBPC were obtained from a standard clinical apheresis.

##### *Purification of CD34<sup>+</sup> cells*

CD34<sup>+</sup> cells were purified using magnetic cell sorting [Miltenyi, 1990 #23] (MiniMACS System, Miltenyi Biotec, Auburn, CA) following the manufacturer's recommendations. For small scale cultures, cells were passed over one LS+ and one MS+ column and eluted with X-Vivo 20

(BioWhittaker, Walkersville, MD). An average of 90.7% of cells were viable as measured by the Trypan Blue exclusion test and average purity was 81.5% as assessed by flow cytometry. For large scale cultures, cells were passed through a CliniMACS System (Miltenyi Biotec) following the manufacturer's recommendations, and eluted with PBS with 2mM EDTA and 1% BSA. An average of 82% of cells were viable as measured by the Trypan Blue exclusion test and average purity was 97% as assessed by flow cytometry. Up to  $10^9$  purified cells from the large scale separation were diluted 1:1 with 20% DMSO (Sigma), and cryopreserved in vapor-phase liquid N<sub>2</sub> until needed (not more than one month).

#### *Large scale culture conditions*

Cells were thawed at 37° for 3 minutes, then transferred to a 50 ml tube containing thawing medium (X-Vivo 20 supplemented with 10 IU/ml heparin (LifeTechnologies) and 10µg/ml DNase (Roche Diagnostics, Basel, Switzerland)). The sample was washed twice in thawing medium and centrifuged at 260xg for 10 min at 4°, then resuspended in 20 ml X-Vivo 20. The sample was injected into a 1L Teflon bag and X-Vivo 20 added to suspend the cells at 400,000 c/ml at an average fluid height of 1cm. Cytokine final concentrations were 100ng/ml Flt-3L, 100ng/ml TPO and 10ng/ml IL-3.

After gently agitating the bag to resuspend the cells, an 3ml aliquot was removed using a 10cc syringe injected into a 7ml Teflon bag, adjusted with bag clip barriers so as to contain a volume sufficient to suspend the cells at an average fluid height of 1cm, for use as a test sample. Samples from this bag were assayed every day for 9 days for total cell count, cell viability, and phenotype expression, via flow cytometry. When cell concentration exceeded  $8 \times 10^5$ /ml, sufficient X-Vivo 20 and cytokines were added to readjust cell concentration to  $4 \times 10^5$ /ml and transferred to additional bags.

#### *Flow cytometric analysis*

Cell aliquots were washed in 1% BSA (Sigma) in PBS (GIBCO) with 5mM EDTA (Sigma), designed to prevent further platelet activation and/or reverse adherence of activated platelets [Dercksen, 1995 #41]. After washing, the cells were stained for 15 min at 4°C in the dark with phycoerythrin-cyanin 5.1 (PC5) conjugated-α-CD34 (Clone 581, Coulter-Immunotech, Miami, FL) and a combination of PE-α-CD41 (Coulter-Immunotech) and FITC-α-CD15 (Coulter-Immunotech), and analyzed by flow cytometry. The negative controls were PC5-, PE- and FITC-α-mouse IgG<sub>1</sub> used at equivalent IgG<sub>1</sub> concentrations. Only the non-apoptotic high forward scatter, low side scatter cell population was used for subset analysis. Samples were not fixed, but analyzed on the same day using 3-color laser (Coulter Epics XL, Coulter Corp, Miami, FL).

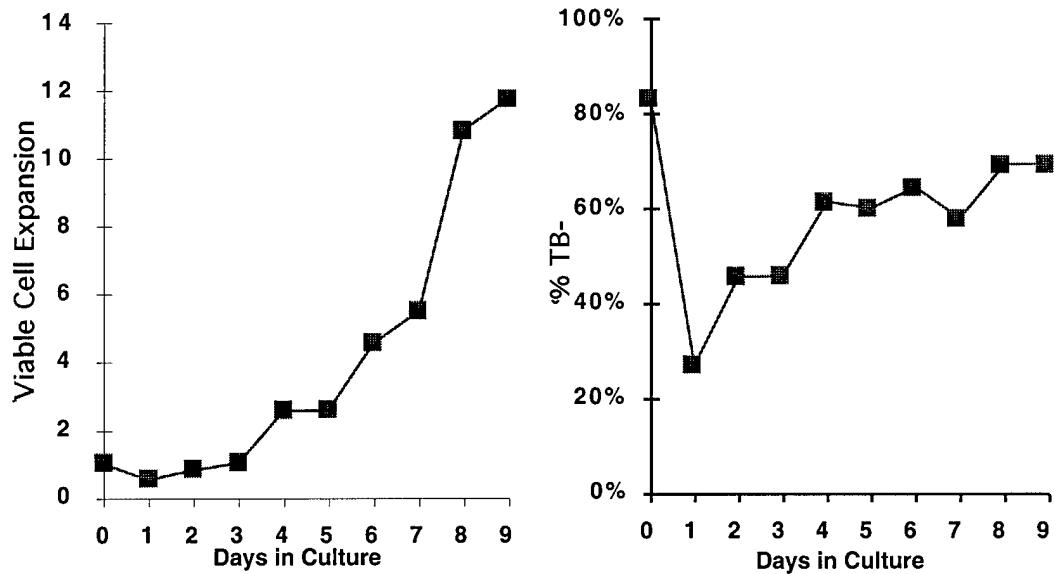
DNA degradation products (<2N DNA), reflecting apoptosis, and megakaryocyte ploidy content were also measured by flow cytometry. FITC α-CD41-stained cells were washed again in 1% BSA-PBS and resuspended in 1mg/ml sodium citrate with 50µg/ml 7-aminoactinomycin D (Sigma), then incubated for >30 minutes at 4°-8°C in the dark [Jackson, 1984 #26]. Samples were analyzed using 3 color laser. All samples were analyzed on the same day.

#### *Clonogenic Assay*

Clonogenic assays using a serum-free collagen medium (MegaCult-C, Stem Cell Technologies, Vancouver, BC) for CFU-MK, and a methylcellulose culture medium (MethoCult GF+, Stem Cell Technologies) for CFU-GM and BFU-E were performed according to the manufacturer's instructions. Viable cells from each day of assay were seeded at  $10^3$ /ml. CFU-MK were scored after ten days and CFU-GM/BFU-E after fourteen days. CFU-MK maturity was evaluated by counting the number of cells in each colony. Four size categories were distinguished: small mature colonies were those with  $\leq 20$  cells, medium-size colonies contained 21-50 cells, large immature colonies contained  $>50$  cells.

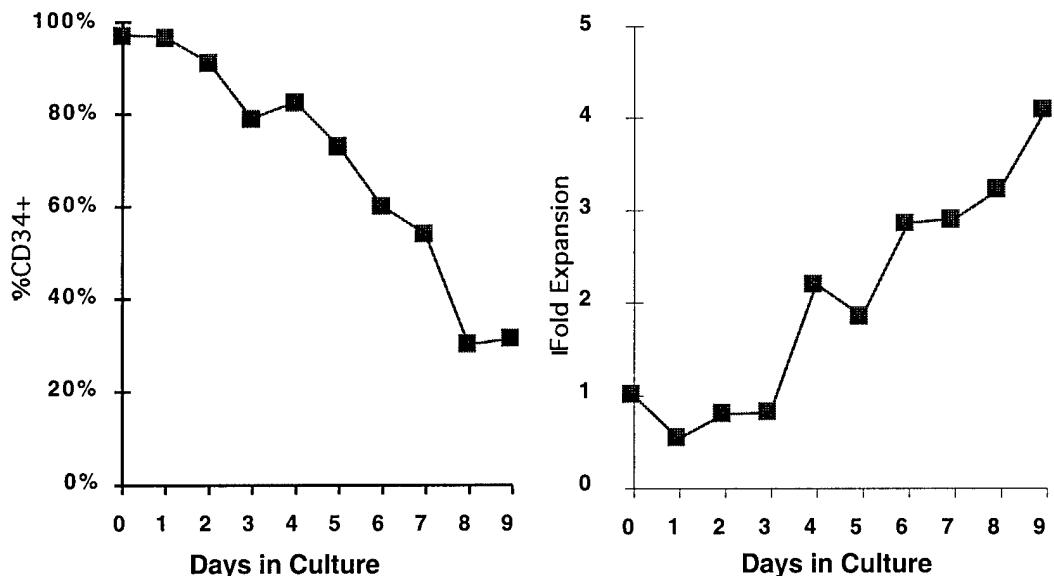
## Results:

### *Cell expansion and viability*



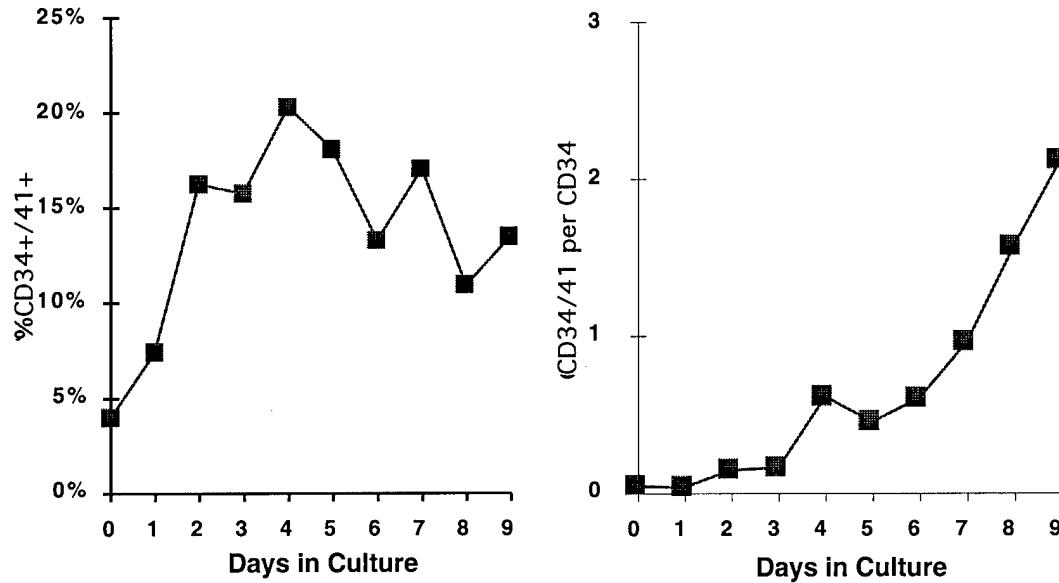
Total viable cells expanded 4.5-fold, with 64% viability after 6 days and 11.7-fold with 69% viability after 9 days.

### *CD34<sup>+</sup> cell expansion*



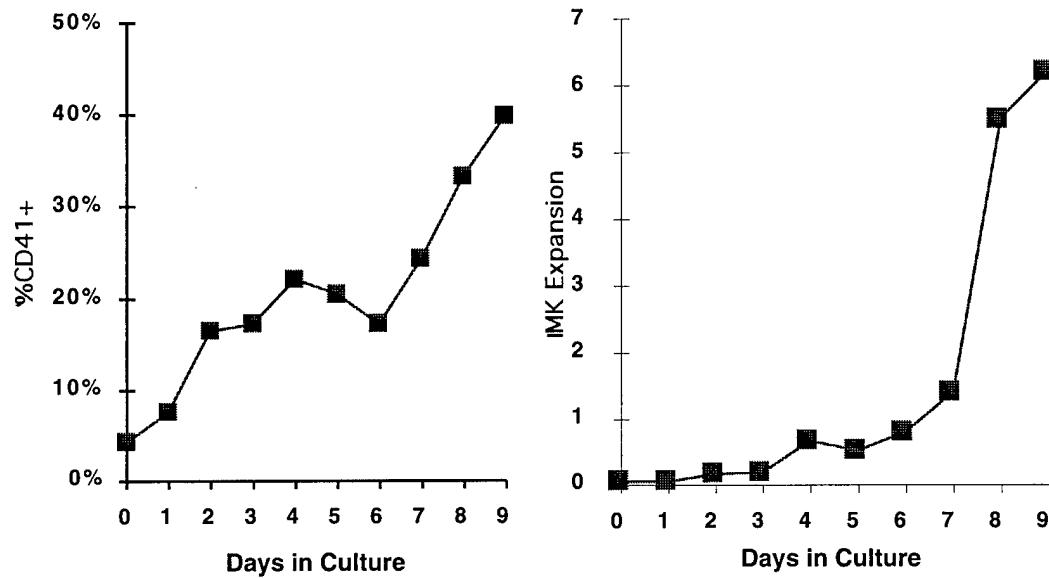
CD34<sup>+</sup> cells declined to 59.9% of total cells at day 6 and 31.3% at day 9, but expanded 2.85-fold and 4.1-fold at day 6 and 9, respectively.

### *Megakaryocyte progenitor expansion*



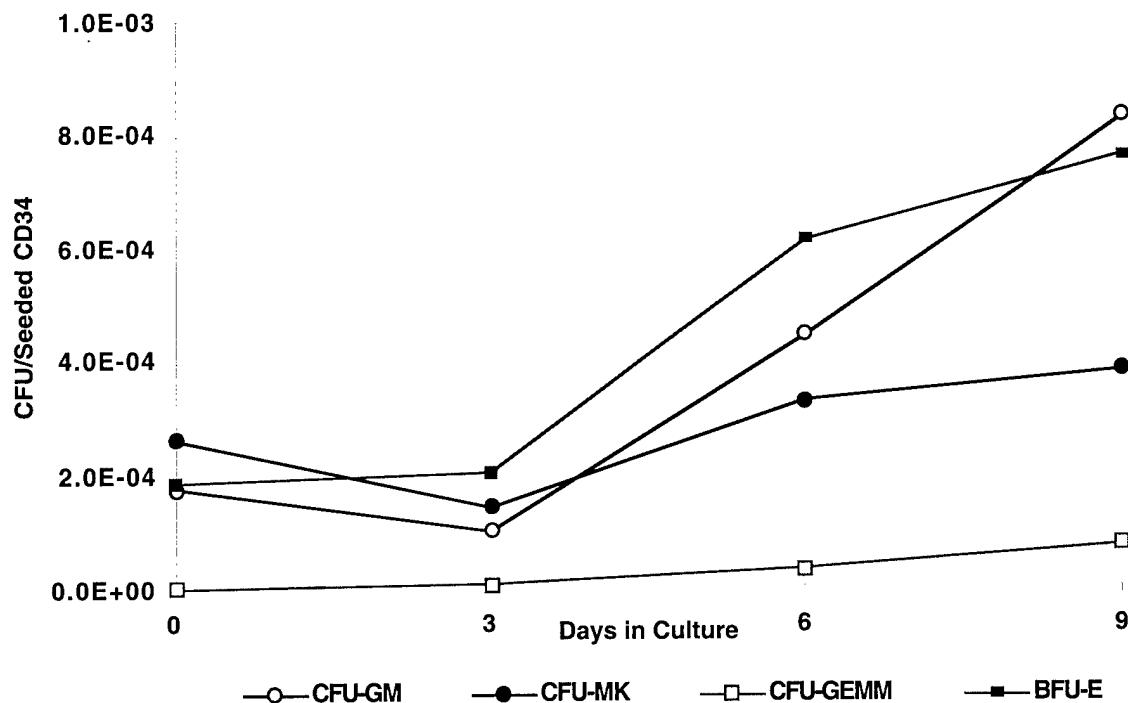
Megakaryocyte progenitors comprised 13.2 and 13.4% of cells at day 6 and 9 respectively, and each seeded CD34+ cell produced 0.6 and 2.1 CD34+/CD41+ cells after 6 and 9 days in culture.

### *Megakaryocyte production*



Megakaryocytes comprised 17.2% and 39.9% of cells at day 6 and 9 respectively, and each seeded CD34+ cell produced 0.8 and 6.2 CD41+ cells after 6 and 9 days in culture.

### Progenitor Colony Assay



All progenitors expanded in the number of colonies produced per seeded CD34+ cell after 6 and 9 days in culture, respectively.

### Discussion and Conclusion:

Our goal is to produce a megakaryocyte-rich product to be used as a supplement to conventional autografts to alleviate post-transplant thrombocytopenia. Our results indicate that the protocol we have developed is capable of producing sufficient megakaryocytes to allow us to transplant up to  $3 \times 10^6$  CD34+/CD41+/kg patient body weight and  $10^7$  MKs/kg patient body weight after 9 days of large scale *ex vivo* culture. This should be more than adequate to test whether this product can meet our goal.

We have shown that the ratio of early MK progenitors versus differentiated megakaryocytes in the *ex-vivo* expanded product changed dramatically over time. As the culture progressed, early progenitor frequencies decreased, but their numbers expanded, driven by total cell expansion. Differentiated megakaryocytes emerged as the MK progenitors matured. Therefore, the maturation distribution of MK in a clinical *ex vivo* expansion protocol will be dependent on the time of cell harvest. The high seeding density used in this study ( $4 \times 10^5$  cells/ml) improved both CFU-MK expansion (by 30% at day 3) and differentiated MK expansion (by 50% at day 13) compared to seeding at only  $10^5$  cells/ml (data not shown). By adjusting the cell concentration as expansion proceeds, we were able to obtain a greater cell number and MK yield without exhausting the culture medium and possibly harming the cells. Our large scale cultures were able to sustain normal viable growth at up to  $1.2 \times 10^6$ /ml before showing signs of media exhaustion, indicating that the serum-free medium we are using is highly optimized for large scale cell expansion.

While it is clear that greater numbers of CD34+/CD41+ cells infused can induce faster platelet engraftment, it can take over a week for even the most rapidly engrafting PB autograft patients to

achieve platelet recovery. *In vivo*, megakaryocytes normally have a 16N modal ploidy, and must reach at least 8N ploidy to produce platelets. *In vitro*, over a week of culture is required for CD34+ cell-derived human megakaryocytes to achieve ploidy greater than 4N and to begin to produce platelets. Since patients infused with even the highest numbers of MK progenitors cannot engraft any more rapidly than a week, this may indicate that early MK progenitors alone may not be sufficient to induce more rapid engraftment. It may be that a longer period (e.g. 6 to 9 days) of *in vitro* culture is required to allow megakaryocytes to mature enough to be able to produce platelets soon after infusion.

Our goal is to clarify whether early megakaryocyte progenitors (6 days culture) or more differentiated megakaryocytes (9 days culture) are more beneficial for rapid platelet recovery. We are beginning our clinical trial to assess the effectiveness of megakaryocytes harvested after different periods of culture and containing different proportions of progenitors and differentiated cells for accelerating platelet engraftment. We hope to show which product is most conducive to accelerating platelet recovery. Our first patient will be enrolled this week (10/8/2001).

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Our large-scale preclinical studies confirmed the results obtained by the small-scale study using TPO, IL-3 and Flt3-L. We can obtain sufficient number of megakaryocytes for alleviating post-transplant thrombocytopenia.
- Approval of IND (BB-IND 9559) by the FDA.
- We are poised to start the clinical trial and have begun to line-up patients eligible for this procedure. We will use mature (9 days culture) and immature (6 days culture) megakaryocytes in two cohorts of patients to determine which is more conducive to produce a large number of platelets which could eliminate or reduce the need for repeated platelet transfusions.

#### **REPORTABLE OUTCOMES:**

- Abstract to the American Society of Hematology (December 2001) - Manuscript in preparation.  
*Lefebvre, P., Winter, J., Cohen, I. Small and large scale ex vivo expansion of early and late megakaryocyte progenitors using TPO, IL-3 and Flt-3L.*

**CONCLUSIONS:** We have determined that the large scale pre-clinical study, using TPO, IL-3 and Flt-3L, is effective in producing sufficient number of megakaryocytes to be used in a clinical setting. We also characterized the features of immature cells, produced after 6 days culture, and mature cells, produced after 9 days culture, which will be used in two cohorts of patients.

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**APPENDICES:**

- 1) Abstract to the American Society of Hematology.
- 2) Synopsis of IND application approved by FDA.
- 3) Quality Monitoring Matrix.

## **Appendix 1**

### **Small and Large Scale *Ex Vivo* Expansion of Early and Late Megakaryocyte Progenitors Using TPO, IL-3, and Flt-3L.**

Phil Lefebvre, Jane Winter, Isaac Cohen. Northwestern University, Chicago, IL.

To alleviate post transplant thrombocytopenia, large scale *ex vivo* expanded human megakaryocytes (MK) may be useful as a supplement to conventional hematopoietic progenitor cell autografts. Small scale cultures were first assayed to determine optimal conditions and potential productivity. Human CD34+ cells, isolated from 4 human bone marrow samples, were seeded at 4 x 10<sup>5</sup> c/ml in serum-free medium with 100ng/ml recombinant human thrombopoietin (TPO) and interleukin-3 (IL-3) at either 1 or 10 ng/ml ± 100 ng/ml Flt-3L ligand (Flt-3L) and characterized after 0, 3, 6, 9 and 13 days by flow cytometry and clonogenic assays. Samples treated with 10ng/ml IL-3 had greater total cell, megakaryocyte and CFU-MK expansion than 1 ng/ml IL-3-treated samples. The addition of Flt-3L further increased total cell, megakaryocyte and CFU-MK expansion. Large scale cultures were initiated using the optimal cytokine combination. CD34+ cells, selected from 4 human peripheral blood mononuclear cells, were seeded at 4.75 x 10<sup>5</sup> c/ml in serum-free medium, with 100 ng/ml TPO and Flt-3L, and 10 ng/ml IL-3. Cells were cultured in gas-permeable Teflon bags at up to 270 ml/bag. A small 7 ml bag was co-cultured and used for daily monitoring, to reduce risk of contamination in the main culture. Cultures were assayed daily for 9 days to monitor progress. When [cell] exceeded 8 x 10<sup>5</sup> c/ml, the cultures were split. No differences were found between the small assay bag and the main large-scale culture. Results were very comparable between the small scale cultures in tissue culture-treated dishes, and the large scale cultures carried out in non-adherent Teflon bags, indicating that the different culture vessels didn't affect culture productivity. After 9 days using the same conditions, small scale cultures expanded 16-fold, large scale cultures expanded 11.7-fold. CD34+ cells expanded 3.2-fold in small scale cultures, and 4.1-fold in large scale cultures. Large scale cultures also produced twice as many CD34+/CD41+ cells per seeded CD34+ cell (2.1 vs 1.0). However, this did not lead to more total CD41+ cells (7.4 vs. 7.4) indicating that the Teflon bags may retard megakaryocyte maturation. This may be due to the non-adherent surface of the bags. The lack of maturation was confirmed by ploidy analysis. Less than 2% of megakaryocytes in the large scale cultures were >4N, while 8.4% were >4N in the small scale cultures. Our goal is to produce high numbers of CD34+/41+ cells from PBPC harvests for use in supplementing conventional autografts. The combination of 100ng/ml TPO and Flt-3L and 10ng/ml IL-3 proved sufficient for expanding CD34+/CD41+ cells. We have found that PB CD34+ cells cultured for 3-6 days are richer in primitive MK progenitors, while those cultured for 9-13 days have greater numbers of more differentiated MKs. The stage of *ex vivo* MK differentiation most conducive to optimal platelet production *in vivo* is not known. We plan a clinical trial to determine the efficacy of *ex vivo* expanded MKs on platelet recovery in relation to MK maturity.

## **Appendix 2**

### **Initial IND Application**

***Ex vivo Expanded Megakaryocytes for Supportive Care of Patients with Breast Cancer or Hematologic Malignancies***

**Jane N. Winter, M.D., PI**

**Isaac Cohen, Ph.D., co-PI**

**Northwestern University Medical School**

**Chicago, Illinois 60611**

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## **3.0 Introductory Statement**

### **3.1 Introduction:**

Patients with breast cancer and hematologic malignancies have been successfully treated with high-dose chemotherapy and transplant of autologous peripheral blood progenitor cells (PBPC). However, most patients experience a two week or more delay in platelet recovery following this treatment. These patients often require platelet transfusions, which are accompanied by great cost, and great risk. To try and alleviate this risk, we propose to augment the conventional stem cell autograft with an *ex vivo*-expanded (EVE) megakaryocyte (MK)-rich hematopoietic cell product, derived from autologous CD34+ selected PBPCs cultured in serum-free medium (X-Vivo 20, BioWhittaker, Walkersville, MD) in the presence of recombinant human thrombopoietin (TPO, R&D Systems, Minneapolis, MN), Flt-3 ligand (Flt-3L, Immunex Corp., Seattle, WA), and interleukin-3 (IL-3, R&D Systems).

MK are the hematopoietic cells responsible for platelet production. Supplementation of conventional PBPC autografts with EVE MK is intended to promote one cycle of autologous platelet production through the critical thrombocytopenic period; long-term platelet engraftment is normally expected to result from reinfusion of unexpanded PBPC. A critical point in the investigation is the determination of the *in vitro* stage of MK development that will be most conducive to platelet production. Evidence that TPO inhibits apoptosis [1] and at high concentration inhibits proplatelet formation [2] supports a possible linkage between platelet formation and programmed cell death. Apoptotic indices may, therefore, help identify the stage of MK development that will result in maximal platelet production.

### **3.2 Description Of Product:**

This biological product is manufactured from one 10 liter, growth factor-mobilized, PBPC apheresis collected from, and produced for, a single patient. The PBPC will be CD34+-cell selected using the CliniMACS (AmCell Corp., Rockville, MD) device. The CD34+ cell-enriched fraction will be cultured in the presence of Flt-3L (100 ng/ml), IL-3 (10 ng/ml), and TPO (100 ng/ml) in X-Vivo 20 serum-free medium for six or nine days. After washing in saline, EVE cells will be resuspended in a citrated human serum albumin (HSA)/saline solution and reinfused through a rapidly flowing central venous catheter on the same day as, but at least two hours after, the conventional autologous PBPC transplant.

### **3.3 Objectives:**

1. To evaluate the toxicity of infusing EVE MK as a supplement to the conventional PBPC autograft in patients undergoing high-dose chemotherapy for the treatment of breast cancer or hematologic malignancies;
2. To assess the effect of EVE MK on platelet recovery and platelet function by comparing treated patients to historical controls matched by protocol, disease status and prior therapy;
3. To compare the frequency of malignant cells in the EVE MK with that of the uncultured PBPC collection;
4. To determine the optimal time of MK harvest for the production of platelets *in vivo*;
5. To determine the required numbers of megakaryocytes for clinical efficacy;
6. To assess the need for multiple transfusions of EVE MK during the post-transplant period.

### **3.4 Previous Human Experience:**

An increasing number of clinical trials investigating the administration of EVE PBPC following high-dose chemotherapy are being reported, and to date, intravenous administration of EVE hematopoietic progenitors, cultured in the presence of a wide variety of cytokine combinations, has not been associated with toxicity [3-10]. Hematopoietic progenitors may be expanded *ex vivo*, while retaining their capacity to reconstitute hematopoiesis [7]. This appears to be true for both cultures optimized for the production of MK and MK precursors [6] as well as for multilineage cultures. Both unselected [8] and CD34+-cell selected [3] PBPC have been used for these studies.

We [11-14] and others have found that great numbers of MKs can be produced *in vitro*, and the rate of productivity is dependent on the culture conditions. The protocol detailed here has been designed to produce sufficient numbers of MKs to allow us to test our objectives.

## **4.0 General Investigational Plan:**

### **4.1 Rationale:**

High-dose chemotherapy with autologous stem cell rescue is an effective approach to the treatment of most hematologic malignancies and some solid tumors that is associated with an obligatory period of pancytopenia. Short-term cultures of autologous PBPC with hematopoietic growth factors including TPO yield large numbers of MK and MK-precursors, especially in the presence of IL-3 and Flt-3L. Supplementation of the conventional autograft with EVE MK is expected to abrogate thrombocytopenia by promoting one cycle of autologous platelet production through the critical thrombocytopenic period.

### **4.2 Indications to be Studied:**

Patients with breast cancer or hematologic malignancies undergoing high-dose chemotherapy followed by autologous stem cell transplant will be eligible for this study.

### **4.3 General Approach:**

- Enrolled patients will proceed to one additional ten liter apheresis, and the CD34+ cell fraction will be collected using a positive selection device.
- The CD34+ cells will be placed in culture in TPO, IL-3, Flt-3L in X-Vivo 20 in one liter Teflon bags to expand MK. The high-dose chemotherapy will be timed so that Day 0 will coincide with the last day of culture.
- As the optimal numbers of days in culture for production of platelets *in vivo* is unknown, patients will be randomized between two groups with different durations of culture ( 6 days vs. 9 days).
- For patients with breast cancer, samples of the pre-culture CD34+ selected cells and the post-culture EVE MK will be studied by immunohistochemistry for the presence of breast carcinoma. For patients with B-cell or T-cell malignancies, semiquantitative PCR will be performed to assess the level of tumor cell contamination pre- and post-culture in an exploratory fashion.
- The number of platelet transfusions, days of thrombocytopenia and numbers of hemorrhagic complications will be monitored. Platelet function studies will be performed at on-study and when the platelet count has recovered to 75,000/ $\mu$ l.
- In the event that a transient rise in platelet count occurs in the first nine days post-transplant, the use of multiple transfusions of EVE-MK will be investigated.

### **4.4 Clinical Trial:**

Women with carcinoma of the breast or patients (men or women) with hematologic malignancies who have met eligibility requirements and signed consent for an active protocol for high-dose chemotherapy with autologous PBPC infusion will be eligible for this study.

### **4.5 Number of Patients:**

24 patients total will be studied, 12 in each arm.

### **4.6 Risks:**

Based on other clinical trials described above and in sections 8 and 9, no significant toxicity related to the infusion of EVE MK is anticipated. Contamination of the product is always a possibility.

Megakaryocyte growth and development factor (MGDF, Amgen Thousand Oaks, CA) is closely related to TPO (Genentech), and has been withdrawn from clinical trial because it was found to be immunogenic. MGDF is a truncated molecule composed of the active N-terminal TPO domain (non-glycosylated) linked to a polyethylene glycol polymer, while the full-length TPO (Genentech) is glycosylated [15]. The Amgen-developed MGDF was found to elicit neutralizing antibodies against endogenous TPO, thereby causing thrombocytopenia [16]. In contrast, TPO developed by Genentech, is being used in a Phase II/III

clinical trial (conducted by Pharmacia) and so far has been found to be safe and non-immunogenic [17]. The TPO provided by R&D for this study has the same molecular structure as the TPO developed by Genentech.

## **5.0 Investigator's Brochure:** Not applicable as described in 21 CFR 312.55.

## **6.0 Protocol:**

### **6.1 Study Protocol:** (See Appendix 1 and 2)

**NU 97B2 Ex vivo Expanded Megakaryocytes for Supportive Care of Patients with Breast Cancer or Hematologic Malignancies.** This protocol (Appendix 1) and consent form (Appendix 2) have been IRB-approved April 20, 2000. Since that time, the sponsoring organization, Department of the Army, U.S. Department of Defense (DoD), has requested some minor changes to the consent form. In addition, the following changes to the protocol have been made:

We have updated the background regarding stem cell transplants in breast cancer (Section 1.1).

Whereas the protocol now includes both patients with hematologic malignancies and breast cancer, Section 1.4 has been changed accordingly. Collection of specimens for anti-TPO antibodies and platelet function is now included in Appendix II, and the methodology for this assay has been added to Section 7.

Whereas the Department of Defense will need to approve these changes and any additional changes requested by the FDA, we have not yet submitted these changes to our IRB, pending the response to this IND application. We will then submit the protocol with any additional changes requested by the FDA to the DoD, and then to our IRB.

### **6.2 Investigator Data:** See Attached Form 1572

### **6.3 Facilities Data:** See Attached Form 1572

### **6.4 Institutional Review Board Data:** See Attached Form 1572

## **7.0 Chemistry, Manufacturing and Controls Information:**

### **7.1 Description of Product**

#### **7.1.1 Description of biological substance:**

Autologous CD34+-selected peripheral blood progenitor cells cultured in TPO, Flt-3L, IL-3, and X-VIVO 20 for 6 or 9 days, then washed in saline and resuspended for infusion in a citrated HSA/saline solution.

#### **7.1.2 Cell type, origin:**

Hematopoietic cells of human origin.

#### **7.1.3 Lot/Batch:**

Each batch constitutes one lot, defined as the progeny of a single PBPC apheresis from a single patient, CD34+ cell selected and expanded *ex vivo*. The product may be contained in a single one liter bag, or expand into two or more bags.

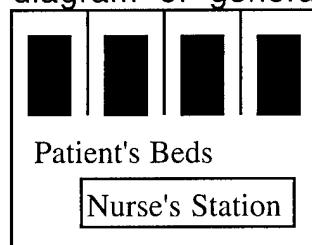
## 7.2 Manufacture

### 7.2.1 Name, address of manufacturer:

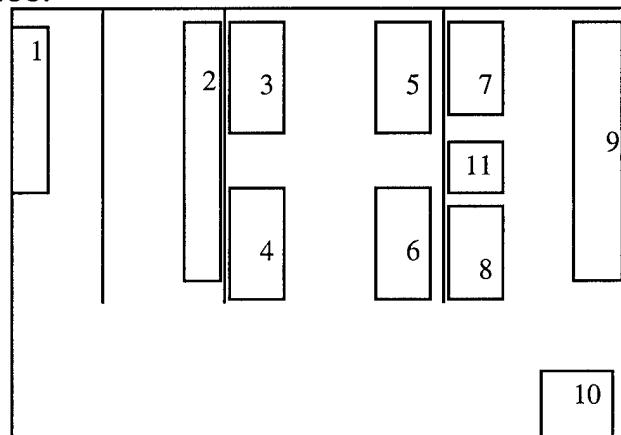
J.N. Winter, M.D., Isaac Cohen, Ph.D.  
Northwestern University Bone Marrow Transplant Laboratory  
Feinberg Pavilion 7-301  
Northwestern Memorial Hospital  
Huron and Fairbanks  
Chicago, IL 60611

### 7.2.2 Floor diagram of general facilities:

Figure 1 A)



B)



#### A) PBPC Collection Site

The cell collection area in the Blood Center of NMH (14th floor of Galter Pavilion). Peripheral blood cell products will be collected from patients undergoing apheresis in this area.

**B) Cell processing and culture site.** The Bone Marrow Transplant Lab of NMH (7th floor Feinberg Pavilion). The Bone Marrow Transplant Lab consists of 4 physically separated areas. The first area is devoted to cell processing procedures, such as cell washing after expansion (1). The next area is office desk space (2), in which data will be analyzed. The third area is designated for inoculation of the cultures using the four laminar flow hoods (3-6). A laminar flow hood (6) has been designated solely for the research procedures described in this application. The final area is where the actual cultures will be incubated. This area has been designated solely for the use of *ex vivo* expansion procedures described in this and other applications (see Section 7.2.3). A refrigerator and -25° freezer is located in this area for reagent storage (7). Plenty of counter space (9) is also available for reagent and/or equipment preparation. A floor model centrifuge (10) is located adjacent to this area, which is involved in various assays as well as plasma preparation. The cells will be incubated at 37°C in a tissue culture incubator (11) dedicated to this study.

### 7.2.3 Additional products to be manufactured in these areas:

*Ex vivo* expanded granulocytic precursors (Separate IND, Dr. Jane Winter, Northwestern University, Chicago, IL).

### 7.2.4 Methods to Prevent Contamination and Cross-Contamination

Any procedure involving the removal of cells, reagents, or materials from sterile, sealed containers will be performed using aseptic technique in a laminar flow hood designated solely for this clinical protocol. The most recent certification of this laminar flow hood (conducted annually) is included in Appendix 3. The specifications and maintenance procedure for the incubator is also included in Appendix 3. All materials (syringes, pipettes, tubing, etc.) used in cell manipulation or reagent preparation in the laminar flow hood will be obtained in sterile, prepackaged form. The specific precautions taken at each stage of the procedure are summarized below:

#### General Contamination Prevention Techniques

- a) The technician will wear clean gloves at all times.
- b) The wash and culture media contain the antibiotic gentamicin.
- c) Cells will be thawed in a sealed plastic bag to prevent seepage from the water bath into the tube.
- d) The sample tube will be wiped with an alcohol wipe before opening.
- e) All samples will be handled in a sterile laminar flow hood when opened to the atmosphere.

- f) Pipets and pipet tips will all contain aerosol barriers to prevent carryover contamination from pipettors.
- g) The samples will be kept in a closed bag, impermeable to organism penetration, in an incubator without any water to avoid atmospheric or water borne contamination.
- h) All samples will be tested for bacterial, fungal and mycoplasmal contamination at 48 hours prior to infusion and at the time of infusion.

### General Cross-Contamination Prevention Techniques

- i) Each culture bag, blood transfer bag, and patient-specific reagents will be clearly labeled.
- j) No two patient samples will be opened at the same time.
- k) Samples will be kept in a closed bag, impermeable to organism penetration, so no outside cells can enter.
- l) No reagent aliquots or disposable supplies will be used with more than one patient sample.
- m) Pipets and pipet tips will all contain aerosol barriers to prevent carryover contamination from pipettors.
- n) Separate laminar flow hoods and incubator chambers designated solely for each protocol will be utilized for products from this protocol and the EVE granulocyte product mentioned in Section 7.2.3.

### Specific Contamination Prevention Techniques for Each Step of Processing

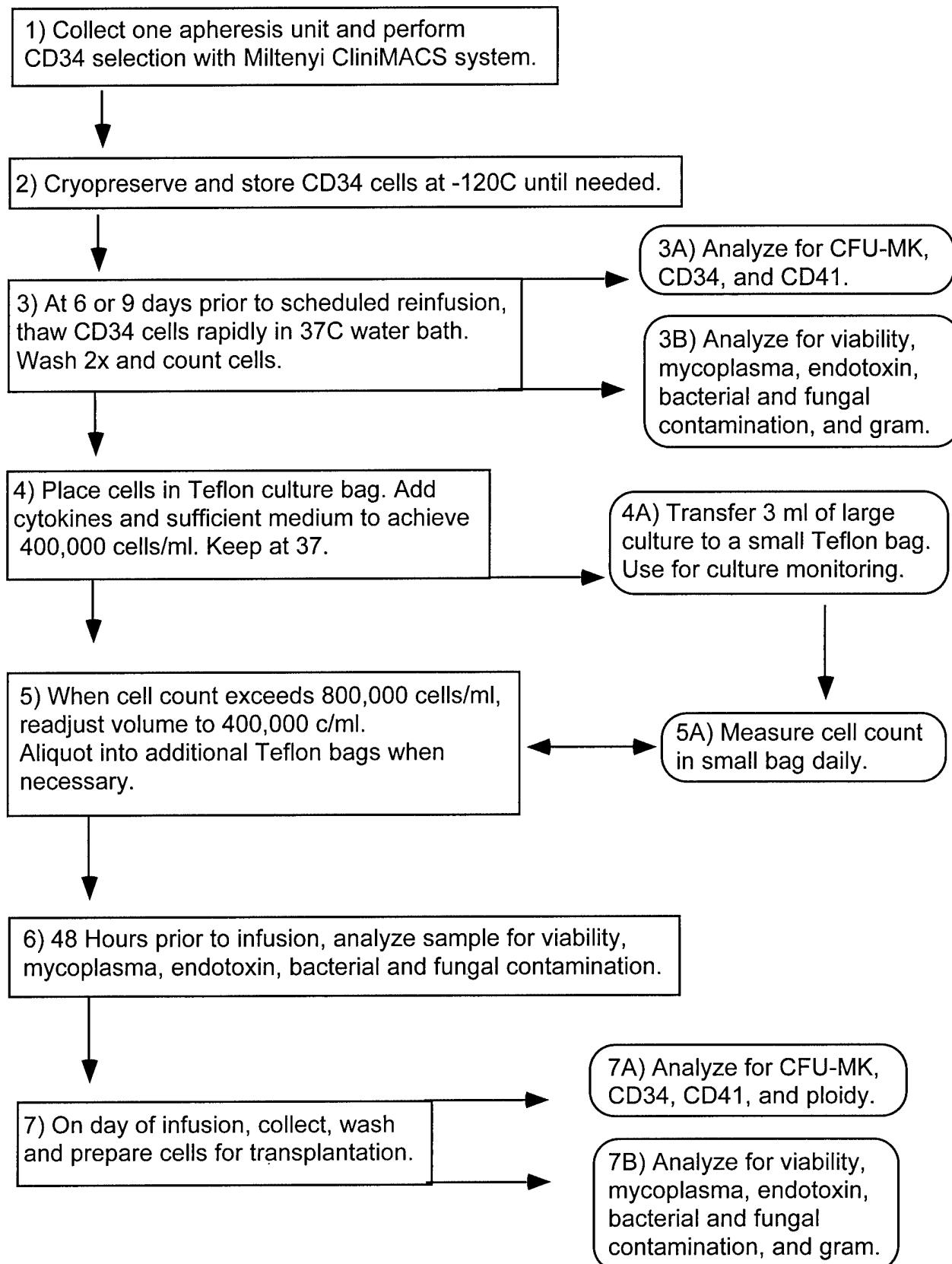
- o) **PBPC Collection** - Mobilized PBPC is obtained in a sterile blood collection bag from the apheresis machine. Removing a fraction of the PBPC for subsequent culture will be conducted in a laminar flow hood using aseptic technique.
- p) **CD34+ Cell Purification** - The CliniMACS machine is a closed system, designed not to allow outside contamination of the cell product. Only sterile reagents and accessories will be used.
- q) **Culture Inoculation** - This entire process takes place in a laminar flow hood using aseptic technique. PBPC, media, and cytokines will be inoculated into 1 liter Teflon culture bags using sterile syringes and tubing components. The culture bags are fitted with Luer-Lok fittings to attach tubing and syringes, to allow leak-free transfer of culture samples. Cultures will be maintained in a standard 37° incubator, which is periodically disinfected.
- r) **Culture Feeding** - Addition of fresh media and cytokines into the Teflon culture bags will be performed with sterile tubing components using aseptic technique in a laminar flow hood.
- s) **Culture Sampling** - Removal of samples for endotoxin, mycoplasma, bacterial and fungal contamination will be performed with sterile syringes using aseptic technique in a laminar flow hood.
- t) **Culture Harvesting** - Transfer of EVE-PBPC from the Teflon bag to sterile blood transfer bags will be performed with sterile tubing components using aseptic technique in a laminar flow hood.
- u) **Cell Washing** - The entire washing procedure occurs in a closed system, in which the harvested EVE-PBPC (contained in sterile blood transfer bags) are concentrated and transferred into another sterile blood transfer bag for patient reinfusion.
- v) **Reinfusion** - PBPC reinfusion takes place under medical supervision using standard aseptic technique.

## 7.3 Method of Manufacture:

### 7.3.1 Manufacturing Process Flow Chart: (Figure 2)

The manufacturing process can be broadly separated into 7 steps, schematically depicted in Figure 2. Briefly, 1) one dedicated ten liter PBPC harvest will be collected and immediately selected for CD34+ cells, then 2) cryopreserved; 3) the sample will be later thawed and suspended in a serum-free medium supplemented with cytokines; 4) the cell suspension will be inoculated into a Teflon bag and cultured for 6 or 9 days; 5) each day a cell count will be taken from a small parallel sample bag. The cell concentration will be readjusted to the initial cell seeding density when the cell count doubles; 6) at day -2, the sample will be assayed for the presence of various types of contaminants; 7) on the day of transplant, the contents of the bags will be harvested, and the final cell product washed and concentrated for infusion.

**Figure 2: Manufacturing Process Flow Chart**



### 7.3.2 Raw Materials and Reagents

An outline of all reagents and vessels that come in contact with the sample is listed below. Relevant support documentation, such as product descriptions, Certificates of Analysis, etc. is compiled in Appendix 3, in the order of that product's appearance in this section.

#### 1. Sample Collection and CD34+ Cell Purification:

COBE Spectra Auto PBPC Pheresis System-Version 6.0	COBE BCT Inc., Lakewood, CO	Approved for Human Use	See Appendix 4
Fenwal CS3000 Plus	Baxter Healthcare Corp. Glendale, CA	Approved for Human Use	See Appendix 4
0.9% Sodium Chloride, USP	Abbott Laboratories, North Chicago, IL	Approved for Human Use	Product Insert, pg. 58
25% Human Serum Albumin, USP	Baxter Healthcare Corp. Glendale, CA	Approved for Human Use	Product Insert pg. 60
CliniMACS System	AmCell Corp., Auburn CA	Not Approved for Human Use	DMF # BB-MF 8061/ MAF 1011, pg 64

#### 2. Cryopreservation:

Autologous Plasma	Collected with patient PBPC	Autologous Use	
Dimethyl Sulfoxide (DMSO)	Edwards Lifesciences, Irvine, CA	Not Approved for Human Use	Certificate of Analysis pg. 67
Medium 199 (M199)	Life Technologies, Rockville, MD	Not Approved for Human Use	Certificate of Analysis pg. 68

#### 3. Thawing and washing of cryopreserved cells

Heparin 5000 Units, USP	Abbott Laboratories North Chicago IL	Approved for Human Use	Product Insert pg. 70
0.22µm Syringe Filters	Gelman Sciences Ann Arbor, MI	Not Approved for Human Use	Product Insert pg. 76
Sterile Water, USP	American Pharmaceutical Partners, Los Angeles, CA	Approved for Human Use	Product Insert pg. 74
DNase 20,000 Units	Roche Molecular Biochemicals Indianapolis IN	Not Approved for Human Use	Product Insert, Cert of Analysis. pg. 72
<ul style="list-style-type: none"> <li>DNase is reconstituted in 10 ml sterile water and sterilized by passing through syringe filters. Solution is stored at -20°C in 1.5 ml aliquots in sterile tubes until needed. Reconstituted solutions expire after 6 months.</li> </ul>			
X-Vivo 20 serum-free medium, GMP grade	BioWhittaker Walkersville MD	Not Approved for Human Use	MDF #BB-MF 2660 pg. 77
<ul style="list-style-type: none"> <li>Thawing Medium is made from X-Vivo 20 supplemented with 10 IU/ml heparin and 10µg/ml DNase.</li> </ul>			

#### 4-5. Cell Culture and Culture Feeding:

VueLife Teflon Bag Culture Systems (1 L and 7 ml)	American Fluoroseal, Gaithersberg, MD	Not Approved for Human Use	Product Description pg. 78
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## **7.4 Component Quality Monitoring** (See Appendix 5)

Testing to be performed on Hematopoietic Progenitor Cells (HPCs). Performance of these tests is designed to assess the quality of HPC components. See Appendix 5.

- **Nucleated Cell Count.** A nucleated cell count is performed for all components after collection and after any subsequent processing. ; SOP # BMT 5.1.02

- **Differential.** After performing a nucleated cell count, place the sample in a biohazard bag, complete the appropriate requisition, and deliver to Hematology for a manual differential. ; SOP # BMT 5.1.02

The expected result for a manual differential is >90% mononuclear cells for PBPC collections.

- **Blood Typing.** The ABO group and Rh type is performed by the Blood Bank on a sample of each HPC component obtained from the donor at collection. If there are previous records, they are compared with the last available record. Any discrepancies must be resolved prior to issue of the component. ; SOP # BMT 5.1.02

- **Cell Viability.** Cells will be analyzed for viability by Trypan Blue stain, and confirmed by flow cytometric light scatter properties. Low viability of the initial seeded cells will not disqualify that product from being cultured, but cultures with less than 50% cell viability will not be transplanted.

- **Flow Cytometry.** The Flow Cytometry laboratory completes a CD34% test after HPC collection and after any subsequent HPC processing. For CD34+ selections and subsequent MK harvests, a battery of tests, including CD34, CD41, CD15 and Ploidy % are completed. SOP # EVE 4

The levels required to proceed with product manufacturing is a minimum of  $1.5 \times 10^5$  viable CD34+ cells/kg patient body weight contained in the peripheral blood progenitor cell harvest, or  $0.875 \times 10^5$  viable CD34+ cells/kg patient body weight obtained after CD34+ selection. Once a product is cultured, the sample will be reinfused, unless it is contaminated.

- **Residual TPO Testing.** A small aliquot of the washed cells will be assayed for residual TPO by ELISA by following manufacturer's direction. (See Quantikine huTPO ELISA, R&D Systems).

## **7.5 Graft Contamination Testing:**(See Appendix 5)

All patients are screened for HbsAg, HIV-1/1, HBC, ALT, STS, HTLV-1, HCV, HIV-1 p24 Antigen, ABO/Rh and Antibody screen, prior to donation. Samples are submitted to LifeSource Blood Services for viral testing within 30 days of the first apheresis, and again on the day of the first apheresis. Also see Appendix 4 SOP # RWC 2.2.01 and SOP # RWC 1.2.07.

### **7.5.1 Microbiology**

A microbiology bone marrow culture is completed by Microbiology. Microbial contamination (bacterial and fungal, and gram stain assay) is monitored after HPC collection and at completion of processing and manipulation. See Appendix 5: SOP # BMT 5.1.02

The expected result for all microbiology samples is negative.

### **7.5.2 Preparation of Culture Sample for Endotoxin and Mycoplasma Testing**

In order to demonstrate safety of an *ex vivo* expanded cell product, freedom from contaminants, such as bacterial endotoxin or mycoplasma, must be demonstrated. Cell product sample is prepared, packaged, and shipped for testing to 2 outside laboratories. Samples will be transplanted if the sample tested 48 hours prior to infusion is negative. See SOP # EVE 2.

### **7.5.3 Immunohistochemical Detection of Breast Cancer Cells in the Product**

A highly sensitive immunohistochemical technique with the capacity to detect one tumor cell in one million mononuclear cells will be used to detect the presence of tumor cells in the peripheral blood progenitor cell collection, and also in the cultured cells [18].

Approximately  $4 \times 10^7$  mononuclear cells from the uncultured apheresis will be cryopreserved in ampules. A similar

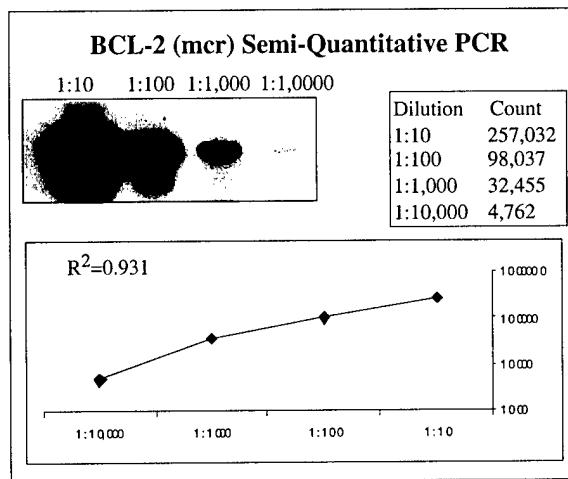
number of cells collected from the *ex vivo* expanded product will also be cryopreserved. After thawing, washing in Iscove's solution and fixing in formalin, the cell samples will be pelleted by centrifugation, dehydrated and infused with paraffin to create a button. Each button will be serially sectioned with approximately eight to ten cross-sections will be mounted on each positively charged slide. The sections will be stained for cytokeratin using a Ventana automated immunostainer. The antibody cocktail AE1/AE3 (Biogenics, Cambridge, MA) will be used. The stain will be developed using an avidin-biotin system and diaminobenzidine as the coloring agent, and counterstained with hematoxylin. Forty-one cross-sections will be examined for each specimen.

In cases of breast cancer, the number of malignant cells detected in forty sections by review of the immunohistochemically stained slides will be counted before and after expansion and the fold-increase will be calculated. A one-sample t-test or a sign test (depending on the distribution of these data) will be used to test that the fold-increase is different from one. For patients with B-cell or T-cell malignancies, a ratio comparing the results of PCR for gene or T-cell receptor rearrangements to a housekeeping gene will be generated, and the relationship between the pre- and post-culture ratios investigated in an exploratory fashion only.

### 7.5.3 Detection Of Malignant Contamination And Expansion During Culture

The polymerase chain reaction may be used to detect small numbers of malignant cells contaminating either a bone marrow or PBPC autograft. Semiquantitative polymerase chain reaction (PCR) for t(14;18) or immunoglobulin (IgH) or T-cell receptor gene rearrangements will be performed on uncultured PBPC, and aliquots of cultured PBPC obtained at the time of harvest to assess whether or not an increase in the number of contaminating malignant lymphoid cells occurs during culture. PCR will be performed on the original lymphoma biopsy specimen, when possible, and on PBPC before and after culturing. PCR for the t(14;18) will be performed on all samples and will be used as the primary assay of lymphoma cell proliferation when possible due to the extreme sensitivity of the assay (detecting one lymphoma cell per 50-100,000 normal cells). PCR for the IgH or TCR- $\gamma$  will be performed on the t(14;18) negative samples as an alternative method for assaying lymphoma cell contamination of the cultures. This approach is significantly less sensitive (detecting only 1 cell per 100 normal cells), but will detect an increase in tumor contamination during culture.

Below we show semi-quantitative PCR for the bcl-2(mcr) with linear regression demonstrating an excellent correlation.



DNA will be isolated from fresh tissue and cultured cells using the Puregene system (Gentra, Minneapolis, MN), and from formalin fixed paraffin embedded samples using the DEXPAT system (Takara, Shiga, Japan). IgH PCR amplification is performed as described in Lehman et al. [19] and for the bcl-2 major breakpoint region (mbr) as described in Crescenzi et al. [20]. PCR for the bcl-2 minor breakpoint cluster region (mcr) is a modification of the protocol found in Ngan et al [21]. For semi-quantitative PCR, a ten-fold serial dilution of the mbr-containing cell line SUDHL-6-DNA or of the mcr-containing cell line SU-DHL-4 DNA in normal DNA is run with the patient samples and densitometry performed on the autoradiogram using a Fluor-S MultiImager (Bio-Rad, Hercules, CA).

## **7.6 In Vivo Testing** (See Appendix 5)

### **7.6.1 Platelet Function Analysis**

To ensure that any platelets produced post transplant are functioning normally, a platelet function analysis will be performed for each patient on the PFA-100 instrument, using 10 ml of citrated whole blood.

### **7.6.2 Platelet Aggregation Assay**

To ensure that any platelets produced post transplant are functioning normally, a platelet aggregation test will be performed for each patient on the PAP-4 System, using 5 ml of citrated plasma.

### **7.6.3 TPO Ab Assay**

Administration of a truncated, non-glycosylated, pegylated form of thrombopoietin (PEG-rHuMGDF) to cancer patients undergoing chemotherapy has been associated with the development of neutralizing antibodies and thrombocytopenia. The time course of anti-TPO antibody titer was inversely related to the platelet count. These results suggest that an IgG<sub>4</sub> autoantibody response developed following PEG-rHuMGDF administration that accounts for the thrombocytopenia. No immunogenic response has been associated with TPO administration. Still, to ensure that the drug does not induce anti-TPO antibodies, patient plasma will be assayed for the presence of anti-TPO antibodies.

A precise method of anti-TPO antibody measurement is available to diagnose and follow such individuals. The method for anti-TPO antibody detection is called RAID (Radio Adsorption Immuno Detection) [22] and is a rapid, sensitive and specific assay for TPO antibody, which has been proven to be very helpful in other clinical trials. RAID will assist in testing for the possible development TPO antibodies in the patients who receive TPO-expanded MK-rich transplants.

For the RAID assay, pre- and post-transplant serum will be collected every 2-3 weeks. Patient whole blood will be collected into 1 ml red top tubes. The serum will be collected by centrifugation and shipped on dry ice to:

Junzhi Li, Ph.D.  
Massachusetts General Hospital  
Jackson 1021  
55 Fruit St.  
Boston, MA 02114

## **8.0 Pharmacology And Toxicology Information**

### **8.1 Animal Studies**

While we have not undertaken any preliminary animal experiments, there have been several studies using *ex vivo*-expanded CD34+ cells in animal models. Ratajczak et. al [23] examined the ability of expanded cells to accelerate platelet recovery in an animal transplant model. Depending on the cytokine combination used, they found that culturing marrow CD34+ cells for 7 to 10 days in serum-free cultures was able to expand CFU-MK approximately 40 to 80 times over input number. Shorter incubation periods were also found to be effective, and when CD34+ cells were exposed to TPO, stem cell factor (SCF), IL-1 $\alpha$  and IL-3 in serum-free cultures for as few as 48 hours, the number of assayable CFU-MK was still increased approximately threefold over input number. The potential clinical utility of this short-term expansion strategy was subsequently tested in an *in vivo* animal model. Lethally irradiated Balbc mice were transplanted with previously frozen syngeneic marrow mononuclear cells (10<sup>6</sup>/mouse), one tenth of which had been primed with TPO, SCF, IL-1 $\alpha$ , and IL-3 under serum-free conditions for 36 hours before cryopreservation. Mice receiving the primed frozen marrow cells recovered their platelet and neutrophil counts 3 to 5 days earlier than mice transplanted with unprimed cells. Mice which received marrow cells that had been primed after thawing but before transplantation had similar recovery kinetics. Thus, pre-transplant priming of hematopoietic cells leads to faster recovery of all hematopoietic lineages, with no adverse or toxic effects noted.

Andrews et. al [24] used a primate model of autologous peripheral blood progenitor cell (PBPC) transplantation to study the effect of *in vitro* expansion on committed progenitor cell engraftment and marrow recovery after transplantation. Four groups of baboons were transplanted with enriched autologous CD34+ PBPC collected by apheresis after five days of G-CSF

administration (100 µg/kg/day). Groups I and III were transplanted with cryopreserved CD34+ PBPC and Groups II and IV were transplanted with CD34+ PBPC that had been cultured for 10 days in Amgen-defined (serum free) medium and stimulated with G-CSF, MGDF, and SCF each at 100 etag/ml. Group III and IV animals were administered G-CSF (100 µg/kg/day) and MGDF (25 µg/kg/day) after transplant, while animals in Groups I and II were not. For the cultured CD34+ PBPC from groups II and IV, the total cell numbers expanded 14.4 +/- 8.3 and 4.0 +/- 0.7-fold, respectively, and CFU-GM expanded 7.2 +/- 0.3 and 8.0 +/- 0.4-fold, respectively. All animals engrafted. If no growth factor support was given after transplant (Groups II and I), the recovery of WBC and platelet production after transplant was prolonged if cells had been cultured prior to transplant (Group II). Administration of post-transplant G-CSF and MGDF shortened the period of neutropenia (ANC 500/µL) from 13 +/- 4 (Group I) to 10 +/- 4 (Group III) days for animals transplanted with non-expanded CD34+ PBPC. For animals transplanted with *ex vivo*-expanded CD34+ PBPC, post-transplant administration of G-CSF and MGDF shortened the duration of neutropenia from 14 +/- 2 (Group II) to 3 +/- 4 (Group IV) days. Recovery of platelet production was slower in all animals transplanted with expanded CD34+ PBPC regardless of post-transplant growth factor administration. Progenitor cells generated in vitro can contribute to early engraftment and mitigate neutropenia when growth factor support is administered post-transplant. Thrombocytopenia was not decreased despite evidence of expansion of megakaryocytes in cultured CD34+ populations.

Most recently, Norol et. al [25] investigated the value of *ex vivo* expanded hematopoietic cells for shortening cytopenia in autologous hematopoietic transplantation. They designed an *ex vivo* expansion protocol based on a cocktail of early acting cytokines and short-term culture and tested it in a baboon model. Expansion involved enriched peripheral blood hematopoietic cells cultured for 6 d with a combination of Flt-3L, SCF, thrombopoietin (TPO) and interleukin (IL)-3 (50 ng/ml each); CD34+ cells, granulocyte-macrophage colony-forming units (GM-CFU) and megakaryocytic colony-forming units (MK-CFU) were amplified, respectively, 10.5-, 20.5- and 17.9-fold. Baboons were submitted to a myeloablative regimen consisting of cyclophosphamide plus total body irradiation (TBI: 6 Gy) and were then grafted with either 2 x 10e6/kg unmanipulated CD34+ cells (control group, n = 4) or cells cultured from 2 x 10e6/kg CD 34+ cells (expansion group, n = 4). No cytokines were administered after transplantation. All the animals engrafted. The mean times to white blood cell (WBC), granulocyte and platelet recovery were significantly shorter in the expansion group than in the control group: WBC (> 1 x 10e9/l) and neutrophil (> 0.5 x 10e9/l) recovery occurred on days 8 (range 6-9) and 9 (range 6-11), respectively, compared with days 12 (range 10-15) and 14 (range 11-16); platelets recovered (> 20 x 10e9/l) on day 9 (range 7-12) compared with day 13 (range 11-15) in the control group ( $P < 0.05$ ). No toxicity was observed after reinfusion. No secondary hypoplasia was observed during more than 12 months of follow-up. Functions of both neutrophils and platelets produced from expanded cells were normal in terms of oxidative metabolism, chemotaxis and the bleeding time. This study shows that in comparison with unmanipulated cells peripheral blood hematopoietic cells expanded from similar doses of CD34+ cells, under the conditions defined here, accelerated both neutrophil and platelet recovery without impairing long-term hematopoiesis.

## 8.2 Small Scale Study Results (Manuscript in Preparation)

### 8.2.1 Summary

**Background:** To alleviate post transplant thrombocytopenia, *ex vivo* expanded human megakaryocytes (MK) may be useful as a supplement to conventional hematopoietic progenitor cell autografts.

**Methods:** CD34+ cells isolated from 4 human bone marrow samples were cultured in serum-free medium with 100ng/ml recombinant human thrombopoietin (TPO) and interleukin-3 (IL-3) at either 1 or 10 ng/ml ± 100 ng/ml Flt-3L ligand (Flt-3L) and characterized after 3, 6, 9 and 13 days by flow cytometry and clonogenic assays. These experiments were preliminary research, and were not carried out using GMP.

**Results:** Samples treated with TPO plus 10 ng/ml IL-3 had greater total cell, megakaryocyte and CFU-MK expansion than TPO plus 1 ng/ml IL-3-treated samples. The addition of Flt-3L further increased total cell, megakaryocyte and CFU-MK expansion.

**Discussion:** Our goal is to produce high numbers of CD34+/41+ cells from PBPC harvests for use in supplementing conventional autografts. The combination of 100ng/ml TPO and Flt-3L and 10 ng/ml IL-3 proved sufficient for expanding CD34+/CD41+ cells. We have previously found that PB CD34+ cells cultured for 3-6 days are richer in primitive MK progenitors, while those cultured for 9-13 days have greater numbers of more differentiated MKs. The stage of *ex vivo* MK differentiation most conducive to optimal platelet production *in vivo* is not known. We plan a clinical trial to determine the efficacy of *ex vivo* expanded MKs on platelet recovery in relation to MK maturity.

## 8.2.2 Materials and Methods

### *Preparation of low density nonadherent mononuclear cells from bone marrow*

All samples were collected within the guidelines of the Northwestern University Institutional Review Board on Human Subjects. Bone marrow, obtained from the femur of four different hematologically normal patients having total hip arthroplasty, was collected in anticoagulant designed to prevent platelet activation and containing final concentrations of 50 IU/ml preservative-free heparin (GIBCO/Life Technologies, Gaithersberg, MD), 1 mM Na<sub>2</sub>EDTA, and 0.1 mg/ml DNase I (Roche Molecular Biochemicals, Indianapolis, IN) in 20 ml α-thioglycerol-free Iscove's modified Dulbecco's medium (IMDM, GIBCO). Marrow cells were repeatedly extracted from bone fragments with IMDM containing 0.1 mg/ml DNase I and 4 µg/ml gentamicin (Life Technologies). The extract was homogenized by passage through a 21 gauge needle to remove bone fragments. Low density mononucleated cells were isolated with the use of Ficoll-Paque (Pharmacia Corp., Piscataway, NJ) as described [26]. Residual red cells were lysed with NH<sub>4</sub>Cl (Sigma) as described [27] and the remaining cells recovered by centrifugation for 6 Min. at 420 x g through a 10% HSA (Baxter Healthcare, Deerfield, IL) in PBS cushion.

### *Purification of CD34<sup>+</sup> cells*

CD34<sup>+</sup> cells were purified using magnetic cell sorting (MiniMACS System, Miltenyi Biotec , Auburn, CA) [28], following the manufacturer's recommendations. Cells were passed over two columns and eluted with X-Vivo 20 (BioWhittaker, Walkersville, MD). An average of 90.7% of cells were viable as measured by the Trypan Blue exclusion test and average purity was 81.5% as assessed by flow cytometry.

### *Culture conditions*

For each cytokine combination, 4x10<sup>5</sup> cells/ml were cultured in X-Vivo 20 serum-free medium (BioWhittaker), with 100 ng/ml recombinant human thrombopoietin (TPO) and either 1 or 10 ng/ml recombinant human interleukin 3 (IL-3) (both from R&D Systems, Minneapolis, MN), with or without at 100 ng/ml Flt-3L (Immunex, Seattle, WA). Cultures were maintained for 13 days at 37°C in a 5% CO<sub>2</sub> fully humidified atmosphere. Cultures were resuspended daily, and cells counted in the presence of Trypan Blue. If cell concentration was ≥ 8x10<sup>5</sup>, it was readjusted to 4x10<sup>5</sup> by diluting with fresh medium and cytokines. Clonogenic and flow cytometric analyses were performed at days 0, 3, 6, 9 and 13. Only the Trypan Blue-negative cells were counted and used for calculating cell proliferation and MK production.

### *Flow cytometric analysis*

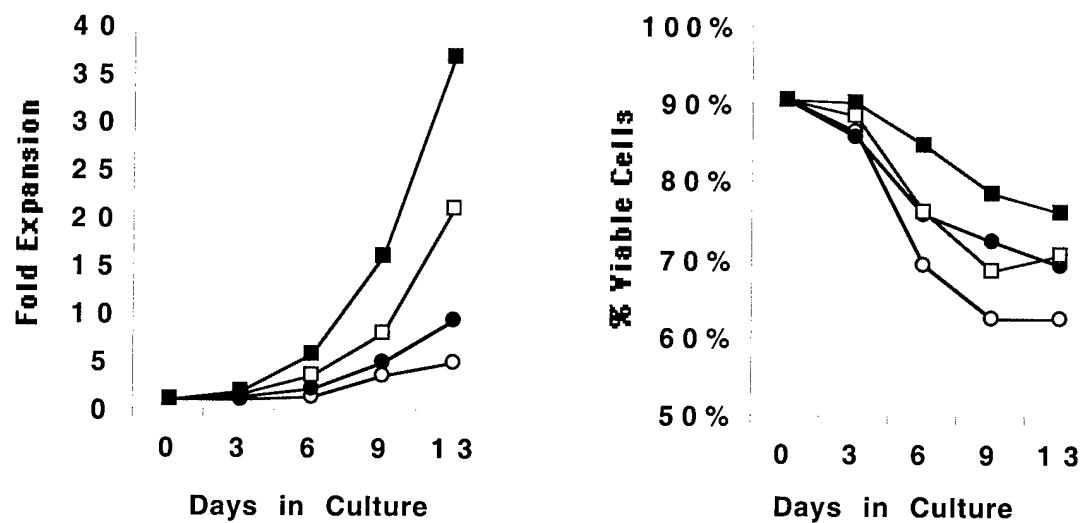
Cell aliquots were washed in 1% BSA (Sigma) in PBS (GIBCO) with 5 mM EDTA (Sigma), designed to prevent further platelet activation and/or reverse adherence of activated platelets [29]. After washing, the cells were stained for 15 Min. at 4°C in the dark with phycoerythrin-cyanin 5.1 (PC5) conjugated-α-CD34 (Clone 581, Coulter-Immunotech, Miami, FL) and a combination of PE-α-CD41 (Coulter-Immunotech) and FITC-α-CD15 (Coulter-Immunotech), and analyzed by flow cytometry. The negative controls were PC5-, PE- and FITC-α-mouse IgG<sub>1</sub> used at equivalent IgG<sub>1</sub> concentrations. Only the non-apoptotic high forward scatter, low side scatter cell population was used for subset analysis. Samples were not fixed, but analyzed on the same day using 3-color laser (Coulter Epics XL, Coulter Corp., Miami, FL).

### *Clonogenic Assay*

A serum-free collagen medium (MegaCult-C, Stem Cell Technologies, Vancouver, BC) was used for CFU-MK, and a methylcellulose culture medium (MethoCult GF+, Stem Cell Technologies) for CFU-GM/BFU-E, performed according to the manufacturer's instructions. Viable cells from each day of assay were seeded at 10<sup>3</sup>/ml. CFU-MK were scored after ten days and CFU-GM/BFU-E after fourteen days. CFU-MK maturity was evaluated by counting the number of cells in each colony. Three size categories were distinguished: small mature colonies were those with ≤20 cells, medium-size colonies contained 21-50 cells, large immature colonies contained >50 cells.

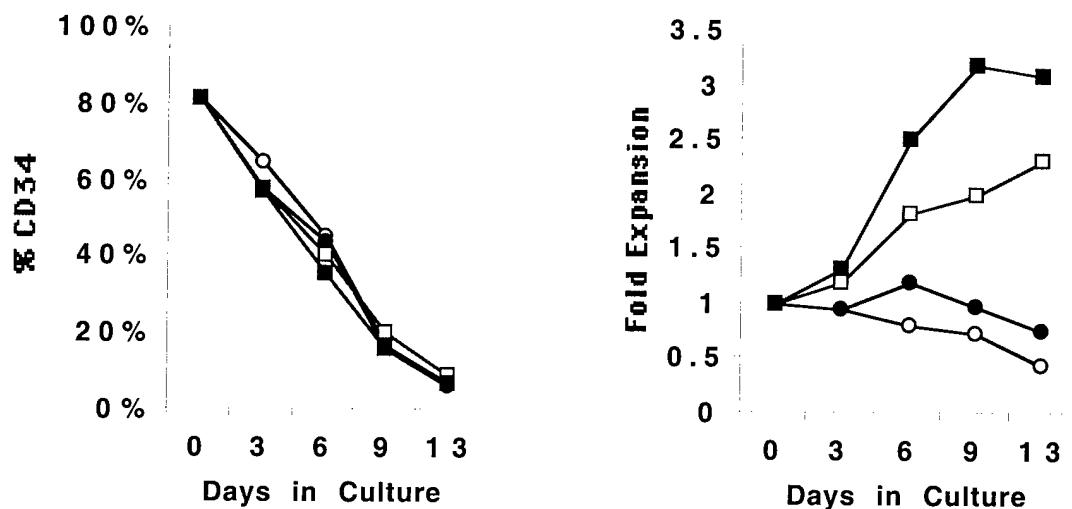
### 8.2.3 Results

#### Total cell expansion



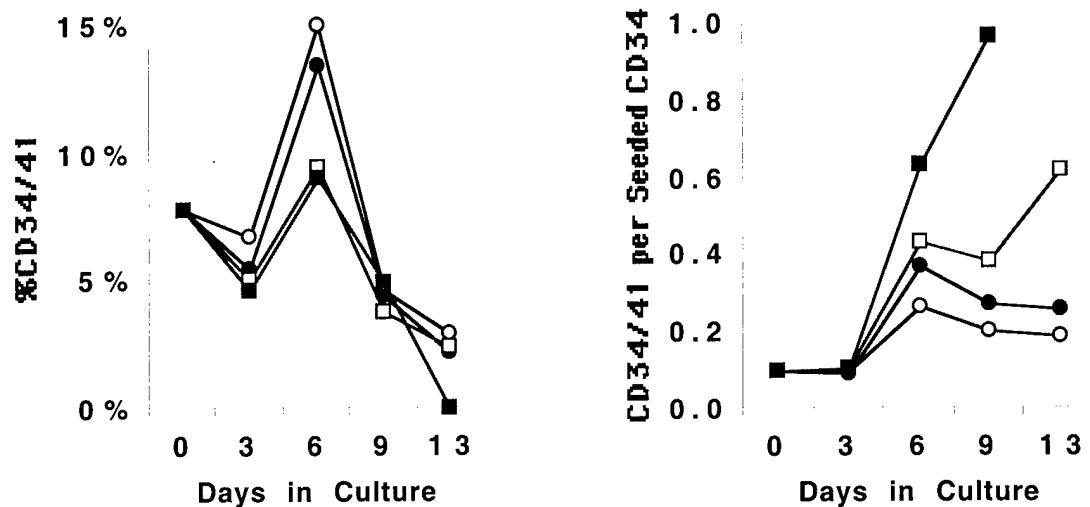
Increasing the IL-3 concentration from 1 ng/ml to 10 ng/ml increases total cell expansion and total cell viability. The addition of Flt-3L also further increased total cell expansion and total cell viability. ■ =TPO + 10 ng/ml IL-3 + Flt-3L; □ =TPO + 1 ng/ml IL-3 + Flt-3L; ● =TPO + 10 ng/ml IL-3; ○ =TPO + 1 ng/ml IL-3.

#### CD34+ cell expansion



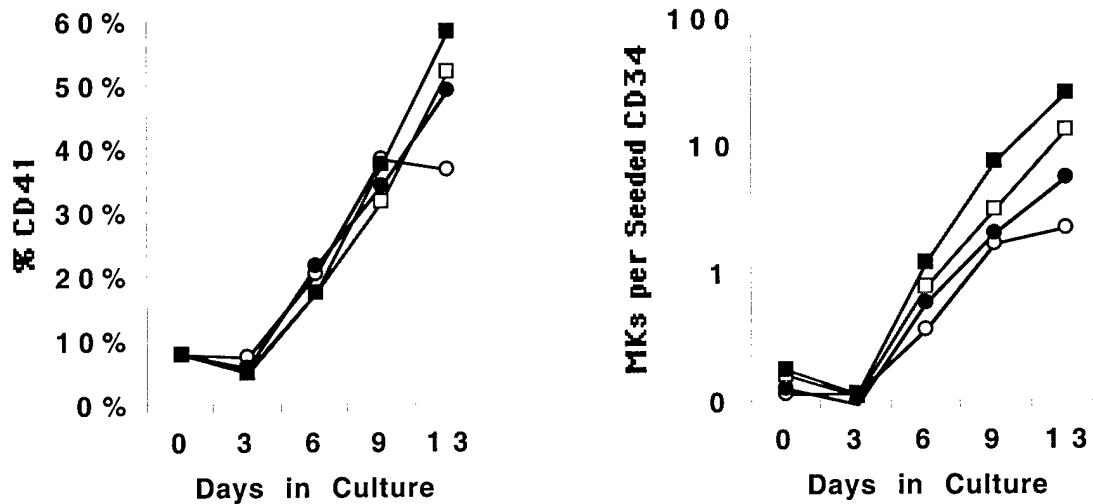
The concentration of IL-3 or the addition of Flt-3L had no effect on CD34+ frequency, but the addition of Flt-3L induced measurable expansion of total CD34+ cells. The use of 10 ng/ml IL-3 further increased CD34+ cell expansion. ■ =TPO + 10 ng/ml IL-3 + Flt-3L; □ =TPO + 1 ng/ml IL-3 + Flt-3L; ● =TPO + 10 ng/ml IL-3; ○ =TPO + 1 ng/ml IL-3.

*CD34/41+ cell expansion*



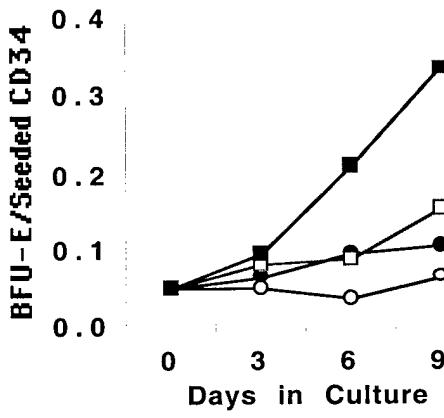
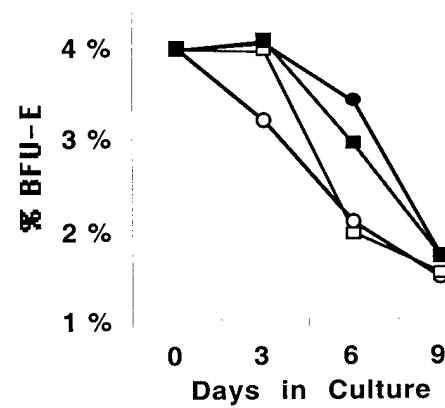
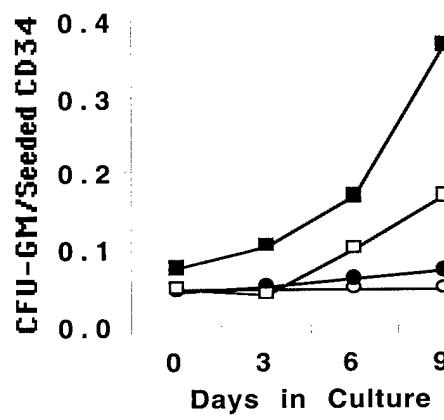
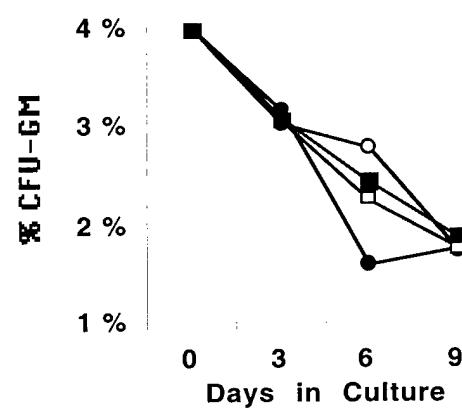
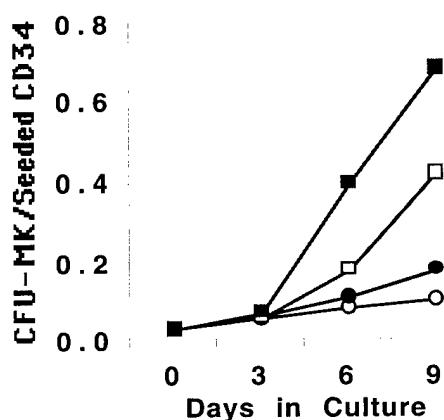
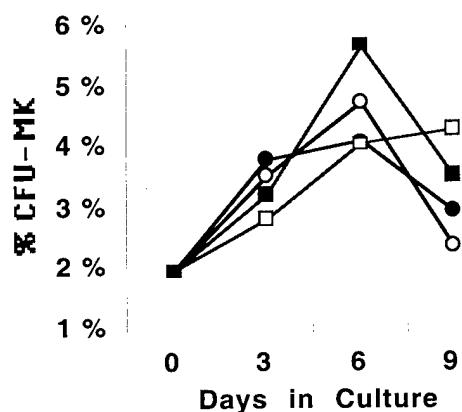
The addition of Flt-3L decreased the frequency of CD34+/41+ cells, but moderately increased the expansion of CD34+/CD41+ cells at day 6 and later. The presence of 10 ng/ml IL-3 greatly increased the expansion of CD34+/CD41+ cells at day 9 ■ =TPO + 10 ng/ml IL-3 + Flt-3L; □ =TPO + 1 ng/ml IL-3 + Flt-3L; ● =TPO + 10 ng/ml IL-3; ○ =TPO + 1 ng/ml IL-3.

*CD41+ cell expansion*



The addition of Flt-3L and increasing the IL-3 concentration to 10 ng/ml induced a moderate increase in CD41 cell expansion. ■ =TPO + 10 ng/ml IL-3 + Flt-3L; □ =TPO + 1 ng/ml IL-3 + Flt-3L; ● =TPO + 10 ng/ml IL-3; ○ =TPO + 1 ng/ml IL-3.

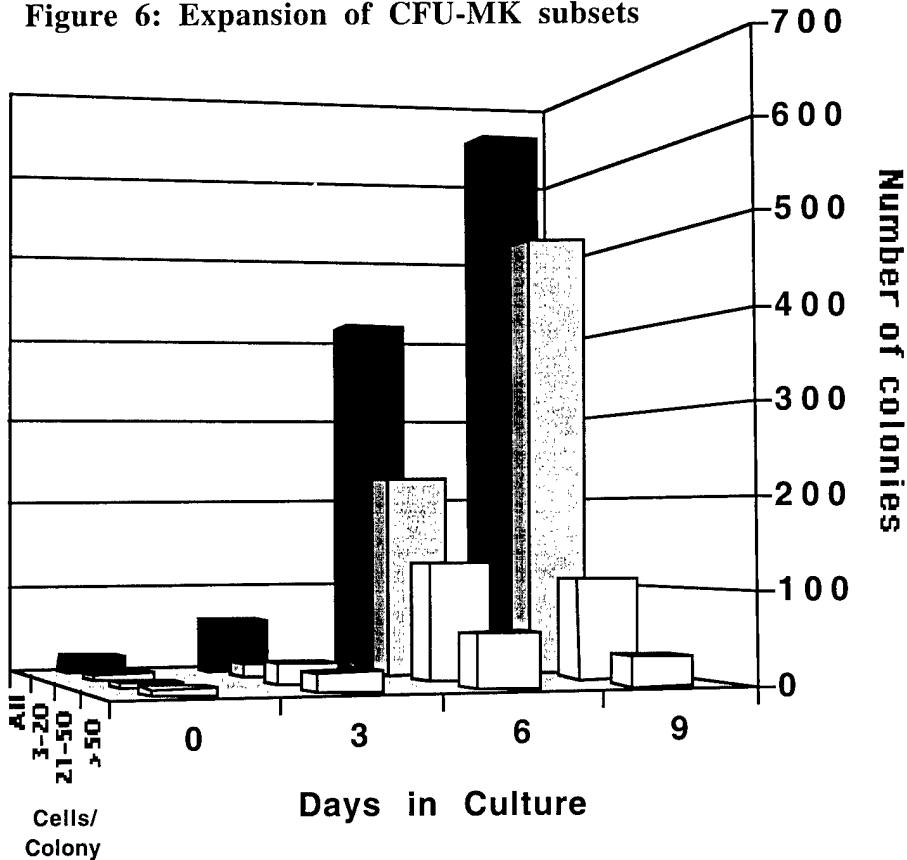
*Colony Forming Unit Cell Expansion*



The addition of Flt-3L increased CFU-MK, CFU-GM, and BFU-E expansion, and increasing the IL-3 concentration to 10 ng/ml in the presence of Flt-3L induced still greater expansion. ■ =TPO + 10 ng/ml IL-3 + Flt-3L; □ =TPO + 1 ng/ml IL-3 + Flt-3L; ● =TPO + 10 ng/ml IL-3; ○ =TPO + 1 ng/ml IL-3.

### *CFU-MK Colony Size Distribution*

**Figure 6: Expansion of CFU-MK subsets**



Immature CFU-MK colonies (> 50 cells/colo ny), and intermediate CFU-MK colonies (21- 50 cells/colo ny) represented 65% and 75% of the total colonies at days 0 and 3 respectively. While the absolute number of immature and intermediate colonies increased at day 6, their relative proportion decreased to 45%, and intermediate colonies were twice as numerous as immature colonies. By day 9 the intermediate and immature colonies represented only 22% of the total CFU-MKs, and intermediate colonies were 3.6-fold more numerous than immature CFU-MK.

## **9.0 Previous Human Experience With EVE PBPC**

An increasing number of clinical trials involving EVE PBPC are being reported, and to date, intravenous administration of EVE hematopoietic progenitors has not been associated with toxicity. This appears to be true for both cultures optimized for the production of MK and MK precursors as well as for multilineage cultures. Both unselected and CD34+ cell selected PBPC have been used for these studies. The cultures have included a wide variety of cytokine combinations.

### **9.1 Unselected PBPC**

Paquette et al. [8] transfused unselected PBPC, cultured for ten days in 1 L Teflon bags with SCF, G-CSF and MGDF, to supplement a conventional autograft in patients with breast cancer. No significant toxicity was associated with infusion of the supplemental *ex vivo* expanded cells.

### **9.2 Selected CD34+ Cells**

Using CD34+ cells, selected from PBPC, Brugger and coworkers [3] cultured a fraction of a typical autograft in medium containing autologous plasma and a cocktail of cytokines (SCF, IL-1 beta, IL-3, IL-6, and erythropoietin; EPO) and infused

this product as the only autograft in ten patients receiving high-dose chemotherapy. Despite the relatively small numbers of cells used to seed the cultures, hematopoietic recovery was similar to that observed in historical controls receiving PBPC or uncultured CD34+ selected cells, with no toxicity. Similarly, Alcorn and colleagues transfused CD34-positive PBPC cultured in autologous serum, SCF, IL-1 $\beta$ , IL-3, IL-6, and EPO, without adverse effects [4]. Additional evidence that hematopoietic progenitors may be expanded *ex vivo*, while retaining their capacity to reconstitute hematopoiesis, is provided by the work of Stiff and colleagues using the Aastrom device to expand a remarkably small volume (40 ml) of bone marrow through coculture with PIXY321, Flt-3L, and EPO [7] CFU-GM were expanded 22.5-fold, and the rate of engraftment was similar to that expected for a bone marrow autograft. Reiffers, et. al [30] safely supplemented a conventional unselected PBPC autograft with CD34+ selected cells that had been cultured for ten days with SCF, G-CSF and MGDF. Using the same growth factor combination and PBPC from patients with breast cancer, McNiece and colleagues observed accelerated engraftment in patients who received expanded cells [10]. There was a significant correlation between the time to neutrophil recovery and the total expanded nucleated cells/kg, but not the CD34+ cell dose/kg. Zimmerman and colleagues expanded CD34+ selected PBPC in the presence of PIXY321 in 21 women with metastatic breast cancer, infusing the expanded product as a supplement to the unselected PBPC autograft [9]. They observed no toxicity associated with the EVE-PBPC infusion and noted a significant inverse relationship between the dose of differentiated myeloid precursors and the depth and duration of neutropenia.

### 9.3 Targeted MK Expansion

A report similar to the goals of this IND application showed no adverse reactions were associated with transfusion of megakaryocytic progenitors generated *ex vivo* in the presence of MGDF, SCF, IL-3, IL-6, IL-11, Flt-3L, and macrophage inflammatory protein -1 $\alpha$  [6]. The patients receiving two of the four highest MK doses did not require platelet transfusions. Our goal is to transfuse a minimum level of MKs equal to the minimum successful number seen in this study.

### 9.4 Other Relevant Works Previously Published

Birkmann, J., et al. (1997). "Effects of recombinant human thrombopoietin alone and in combination with erythropoietin and early-acting cytokines on human mobilized purified CD34+ progenitor cells cultured in serum-depleted medium." [31]

The effects of recombinant thrombopoietin (TPO) alone and in combination with erythropoietin (EPO) and early-acting cytokines such as interleukin 3 (IL-3), stem cell factor (SCF) and GM-CSF on highly purified mobilized human CD34+ progenitor cells were studied in a serum-depleted culture system. Eight leukopheresis samples were cultured for seven days and analyzed; aliquots were re-plated and re-evaluated on day 12. Three-color flow cytometry was used together with morphologic analysis to determine proliferation and megakaryocytic or erythroid maturation. TPO alone was sufficient for cell survival and proliferation in serum-depleted medium. In the absence of other growth factors, almost all CD34+ cells differentiated along the megakaryocytic pathway within 12 days. Concomitantly, the progenitor cells gradually acquired the morphologic features of mature megakaryocytes. After exposure to TPO for one week, 50% of the cells still expressed CD34; by day 12 the remaining CD34+ cells (11%) were all coexpressing CD41. TPO alone did not support proliferation of glycophorin-A-positive cells. The addition of TPO to early-acting cytokines (EPO, GM-CSF, SCF and/or IL-3) not only increased the overall megakaryocyte expansion, but also generated a different maturation pattern of the CD41+ megakaryocyte progenitors. It further doubled the number of erythroid cells and C-kit+ cells in the second week of culture. Interestingly, the overall number of CD34+ cells was increased about fivefold when TPO was added to the early-acting cytokines, with a marked expansion of the CD34+/CD41+ and CD34+/CD117+ subpopulations. TPO can augment the pool of committed progenitors, thereby increasing the number of its own target cells and the number of EPO-responsive cells. These properties make TPO an interesting cytokine for the *ex vivo* expansion of human progenitor cells.

Briddell, RA., et al. (1997). "Purification of CD34+ cells is essential for optimal *ex vivo* expansion of umbilical cord blood cells." [32]

Allogeneic umbilical cord blood (UCB) cells have recently been used for transplantation following high-dose chemotherapy. However, the numbers of total cells, including progenitor cells, harvested are low compared with bone marrow or peripheral blood progenitor cell harvests. Therefore, we evaluated the potential of UCB cells for their ability to expand granulocyte-macrophage colony-forming cells (GM-CFC) and burst-forming unit-erythroid (BFU-E) cells over 10 days. We used an ammonium chloride lysing buffer to eliminate the majority of contaminating red blood cells. An average recovery of 61% of the starting number of white blood cells was obtained, while retaining

100% of the CD34+ cells. *Ex vivo* expansion cultures were established in Teflon cell culture bags (American Fluoroseal Corp., Columbia, MD) in 25 ml defined medium (Amgen Inc., Thousand Oaks, CA) containing 100 ng/ml each of stem cell factor (SCF), granulocyte colony-stimulating factor (G-CSF), and megakaryocyte growth and development factor. Either unselected UCB cells or CD34+ UCB cells, selected with Magnetic Activation Cell Sorting technology (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), were incubated for 10 days at 37 degrees C without refeeding. Unselected UCB cells seeded at  $1 \times 10^6$ /ml produced an average expansion of 1.4-fold in total cells, 0.8-fold in GM-CFC, and 0.3-fold in BFU-E cells. By contrast, CD34+ selected UCB cells seeded at  $1.0 \times 10^4$ /ml produced an average expansion of 113-fold in total cells, 72.6-fold in GM-CFC, and 49-fold in BFU-E cells. These data demonstrate that CD34+ cell selection is necessary for optimal expansion of both GM-CFC and BFU-E cells. The cell numbers thus obtained postexpansion may be sufficient for transplantation in adults.

Gehling, UM., et al. (1997). "Ex vivo expansion of megakaryocyte progenitors: effect of various growth factor combinations on CD34+ progenitor cells from bone marrow and G-CSF-mobilized peripheral blood." [33]

Prolonged thrombocytopenia resulting from inadequate megakaryocyte (MK) progenitor cell reconstitution is a serious complication of hematopoietic cell-supported high-dose chemotherapy (HDC). In this situation, the infusion of MK progenitors that are expanded *ex vivo* could be clinically beneficial. In this study we investigated the ability of various growth factor combinations to generate MK progenitors. CD34+ cells derived from bone marrow (BM) and granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood (PB) from 17 patients with breast cancer, lymphoma, or myeloma were cultured unperturbed for 10 days in a serum-free liquid culture system that contained recombinant growth factors. Five different growth factors combinations were evaluated: Stem cell factor (SCF), interleukin (IL)-3, IL-6 + G-CSF (combination 1); SCF, megakaryocyte growth and development factor (MGDF) + G-CSF (combination 2); SCF + MGDF (combination 3); MGDF alone (combination 4); and SCF, IL-3, IL-6, G-CSF + MGDF (combination 5). PB CD34+ cells yielded significantly higher numbers of CD41+ MK progenitors than BM CD34+ cells with any of the growth factor regimens assayed. PB CD34+ cells ( $2 \times 10^5$ ) at day 0 generated 1.2 to  $1.3 \times 10^6$  CD41+ cells by day 10 when cultured in the presence of growth factor combinations 1, 2, or 3. In contrast,  $2 \times 10^5$  BM CD34+ cells produced  $5 \times 10^5$  CD41+ cells after 9 days in the presence of combination 1, whereas lower numbers of CD41+ cells were generated in cultures with combinations 2 and 3 ( $2.3 \times 10^5$  and  $4.2 \times 10^4$ , respectively). The addition of MGDF to cultures that were grown with combination 1 for 5 days increased the number of CD41+ cells (1.7-fold increase in PB-derived cultures, 1.6-fold increase in BM-derived cultures). Treatment with MGDF alone resulted in higher frequencies of MK progenitors than those obtained in cultures with combined growth factors (79% in PB-derived cultures, 25% in BM-derived cultures), but because total cell growth was attenuated, absolute numbers of MK progenitors were lower ( $7 \times 10^5$  in PB-derived cultures,  $7 \times 10^4$  in BM). Morphological analysis of immunocytochemically identified megakaryocytic cells revealed mononuclear cells as the predominant cell type in all of the cultures. During the 10-day culture period, PB-derived MK progenitors did not show notable maturation, even under the influence of MGDF, whereas in BM-derived cultures MGDF induced a significant shift to binuclear cells and stage I MK after day 5. Phenotypic analysis of cell surface markers showed that the majority of cultured megakaryocytic cells coexpressed CD34 and platelet glycoproteins (GPs), also indicating an immature stage of development. The *ex vivo* proliferative activity of CD34+ cells and their potential to develop into the megakaryocytic lineage demonstrated considerably high interpatient variations. There was no correlation between platelet recovery following HDC with hematopoietic cell support and the magnitude of GP+ cell expansion *ex vivo*, suggesting the feasibility of MK expansion *ex vivo* in patients with prolonged thrombocytopenia post-transplantation. In summary, these data indicate that G-CSF-mobilized CD34+ PBPCs are more effectively expanded *ex vivo* into the megakaryocytic lineage than are CD34+ BMPCs. CD34+/GP+ MK progenitors may be an appropriate cell population for transplantation as prophylaxis or treatment of prolonged thrombocytopenia. The efficacy of this procedure will be tested prospectively in a clinical trial.

Halle, P, et al. (2000). "Ex vivo expansion of CD34+/CD41+ late progenitors from enriched PB CD34+cells." [34]

In our experience, patients with neuroblastoma who undergo transplantation with CD34+ cells following high-dose chemotherapy have prolonged delays in platelet recovery. In vitro expansion of megakaryocyte (MK) cells may provide a complementary transplant product able to enhance platelet production in the recipient. We investigated the ability of a combination of various hematopoietic growth factors to generate *ex vivo* MK progenitors. Immunoselected CD34+ cells from peripheral blood stems cells (PBSCs) were cultured in media with or without serum, supplemented by IL-3, IL-6,

IL-11, SCF, TPO, Flt-3 ligand, and MIP-1 alpha. In terms of MK phenotypes, we observed a maximal expansion of CD61+, CD41+, and CD42a of 69-, 60-, and 69-fold, respectively, i.e., 8-10 times greater than the expansion of total cell numbers. Whereas the absolute increment of CD34+ cells was slightly elevated (fourfold) we showed increases of 163-, 212-, and 128-fold for CD34+/CD61+, CD34+/CD41+, and CD34+/CD42a+ cells, respectively. We obtained only a modest expansion of CFU-MKs after only 4 days of culture (fourfold) and similar levels of CFU-MKs were observed after 7 days (fivefold). Morphology and immunohistochemistry CD41+ analyses confirmed expansion of a majority of CD41+ immature cells on days 4 and 7, while on day 10 mature cells began to appear. These results show that primarily MK progenitors are expanded after 4 days of culture, whereas MK precursor expansion occurs after 7 days. When we compared the two culture media (with and without serum) we observed that increases of all specific phenotypes of the MK lineage were more elevated in serum-free culture than in medium with serum. This difference was especially marked for CD34+/CD61+ and CD34+/CD41+ (163 vs. 42 and 212 vs. 36, respectively). We contaminated CD34+ cells with a neuroblastoma cell line and we observed no expansion of malignant cells in our culture conditions (RT-PCR for tyrosine hydroxylase positive at day 4 and negative at day 7). With our combination of hematopoietic growth factors we are able to sufficiently expand *ex vivo* MK late progenitor cells to be used as complementary transplant products in neuroblastoma patients who undergo transplantation with CD34+ cells. It is possible that these committed MK late progenitors could accelerate short-term platelet recovery in the recipient until more primitive progenitor cells have had time to engraft.

Hunnestad, JA., et al. (1999). "Thrombopoietin combined with early-acting growth factors effectively expands human hematopoietic progenitor cells in vitro." [35]

Thrombopoietin (TPO) is established as a powerful stimulant of megakaryocyte differentiation and platelet production both *in vivo* and *in vitro*. In preparation for future transplantation of *ex vivo* expanded CD34+ hematopoietic progenitor cells (HPCs), we have examined the *in vitro* effect of TPO on cultures of HPC when combined with other early-acting hematopoietic growth factors (GFs) in an attempt to decrease post-transplant thrombocytopenia and accelerate engraftment. By adding TPO to all possible combinations of GM-CSF, IL-3, and c-kit ligand (CKL) in a suspension culture system, we found a significant increase in both relative and absolute numbers of cells in cultures containing TPO of the megakaryocytic lineage and CD34+ cells after 14 days of culture. The most efficient GF combinations for expansion of cell populations of the megakaryocytic lineage and HPCs were TPO, GM-CSF, and CKL, which increased the number of cells of the megakaryocytic lineage 78 fold and the number of CD34+ cells 1.8 fold. The number of CD34+ cells decreased in the cultures containing GM-CSF and CKL with no TPO present, and the number of cells of the megakaryocytic lineage was increased merely 27 fold. Based on our findings, we suggest adding cells from HPCs expanded in cultures containing TPO, GM-CSF, and CKL to unexpanded stem cells for stem cell transplantation.

Linker, C, et al. (1998). "A Randomized, Placebo-Controlled, Phase II Trial Of Recombinant Human Thrombopoietin (rhuTPO) In Subjects Undergoing High Dose Chemotherapy (HDC) and PBPC Transplant." [36]

This study evaluated the activity and safety of rhuTPO in two settings: 1) pre-transplant: to increase PBPC mobilization when administered with G-CSF 2) post-transplant: to accelerate platelet (plt) recovery. 87% of the 134 randomized patients (pts) were women with breast cancer without extensive prior therapy median age 48 years. Pts were randomized to receive rhuTPO 1.5 µg/kg IV by one of three schedules: 1.5 µg/kg on Mobilization Day (MD) 5 (Grp 1) 1.5 µg/kg on MD1 (Grp 2) 0.5 µg/kg on MD1, 3 and 5 (Grps 3 and 4) control - placebo (Grp 5). Daily G-CSF (10 µg/kg/day) began on MD5, and leukapheresis on MD9, continuing until a target graft of  $5 \times 10^6$  CD34+ cells/kg was collected or a maximum 6 aphereses. Pts with a minimum PBPC graft of  $2 \times 10^6$  CD34+ cells/kg went on to transplant after a site-specific HDC regimen. Post-transplant, Grps 1-3 and 5 received G-CSF 5 µg/kg/day and Grp 4 received GM-CSF 250 µg/m<sup>2</sup>/day. Post-transplant Grps 1-4 also received rhuTPO at 1.5 µg/kg IV on RD 0, 2, 4, and 6. Grp 5 received placebo. 129 pts received study drug (SD) and underwent 1 apheresis, 115 achieved the minimum graft, and 112 were transplanted receiving SD post-transplant. Results are outlined in Table 1:

Table 1	:Endpoint (median)	Grp 1	Grp 2	Grp 3	Grp 4	Grp 5	P-value
n =	27	27	26	21	28		
Avg. CD34+ cells ( $\times 10^6$ /kg/Apheresis)	1.86	2.67	3.10		1.65	0.006	
% Achieving Target	67%	70%	79%		46%	0.041	
(Minimum) Graft	(85%)	(96%)	(94%)		(75%)	0.050	
# Phereses to Collect Target Graft	3.0	2.0	2.0		4.0	0.015	
Day of Plt Recovery to 20,000/ $\mu$ L	10.5	10.0	10.0	10.0	10.0	0.815	

All groups had rapid neutrophil engraftment between RD9 and 11. rhuTPO had no effect on post-transplant plt recovery in pts receiving an adequate stem cell dose. Post-transplant, there was a higher incidence of rash (predominantly grade I-II) in Grps 1-4 (52%) than Grp 5 (24%). There was no significant difference between the arms with respect to the incidence of bleeding and clotting events, or other adverse events. No anti-TPO antibodies were observed. In this study, rhuTPO was found to be safe and well-tolerated. rhuTPO administered with G-CSF increased CD34+ cell harvests two-fold and reduced the number of aphereses required to collect a target PBPC graft. These data support a Phase III study to demonstrate the clinical benefit of rhuTPO, when administered with G-CSF for PBPC mobilization.

Nash, R, et al. (1997). "Safety and activity of recombinant human TPO in patients with delayed platelet recovery." [37].

**Introduction:** Myeloablative therapy with hematopoietic stem cell transplantation (HSCT) is associated with pancytopenia, which is self-limited in most pts. Neutrophil recovery may be enhanced by G-CSF or GM-CSF, but 10-20% of pts have DPR (platelet (plt) transfusion-dependence at 35 days post-transplant). Unfortunately, commercially available cytokines have had no impact upon plt recovery. This study was conducted to determine if rhuTPO could safely increase the probability of achieving plt transfusion independence (plt count 20 K/ml without transfusions) in this patient population.

**Methods:** Eligible pts must have received an autologous or allogeneic graft (BM or PBPC) 35, but 90 days prior to study entry and been unable to maintain a plt count 20 K/ml without transfusions for 7 days prior to study entry. Additional entry criteria included an ECOG performance status 2: adequate end-organ function, no prior bleeding diathesis or clotting abnormalities, age 16 y. o., no progressive malignancy or GVHD, and informed consent. G-CSF was given if clinically indicated. Pts received IV rhuTPO in escalating cohorts of 6 each in single and multiple doses of 0.6-2.4 mg/kg.

**Results:** 38 pts were enrolled, 25 males & 13 females. The median age was 48 yo. (23-59 years). The diagnoses were: leukemia or myelodysplasia (n= 21), non-Hodgkin's lymphoma--(11) breast cancer (4) other (2). 11/22 allogeneic grafts were from matched unrelated donors 11/16 autologous grafts were PBPCs. BM examination (n= 37) revealed megakaryocyte hypoplasia in all at baseline 3/37 pts had increases in marrow megakaryocytes 1 month after dosing with rhuTPO. Only 2/38 pts achieved plt transfusion independence during the study period both received single doses of rhuTPO at 2.4 mg/kg. The median endogenous TPO level before dosing was elevated to 1.39 ng/ml (range = 0.64-9.47 ng/ml). Increases in serum TPO levels were dose-dependent, with nearly 100-fold increases in the serum TPO levels achieved at the highest dose levels. 256 plt transfusion events were recorded during the 28-day study period (median = 6 range = 0-20). There was no evidence of a dose-response on the probability of transfusion independence or number of plt transfusions observed. rhuTPO was not associated with any serious adverse events. No neutralizing anti-TPO antibodies were observed one subject was noted to have non-neutralizing antibodies which bound TPO before receiving any rhuTPO, without any discernible effect.

**Conclusions:** The administration of rhuTPO for DPR after HSCT was safe but had limited clinical activity.

Piacibello, W., et al. (1996). "The effects of human Flt-3 ligand on in vitro human megakaryocytopoiesis." [38]

The human homolog of the murine flt3/flk2 gene product is a tyrosine kinase receptor that plays a role in regulating the proliferation and differentiation of cells in the hematopoietic system. Using a plasma-clot clonal assay and a long-term bone marrow culture (LTBMC) system, we studied the effects of the recently cloned human Flt-3 ligand (FL) alone and in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), or stem cell factor (c-kit ligand, KL) on human megakaryocytopoiesis. The effects of FL on the primitive megakaryocyte (MK) progenitor cell, the burst-forming unit-megakaryocyte (BFU-MK), and the more differentiated colony-forming unit-megakaryocyte (CFU-MK) were determined. FL alone had no megakaryocytic

colony-stimulating activity (MK-CSA), but was capable of augmenting the MK-CSA of both GM-CSF and IL-3. FL synergized with IL-3 at the level of both CFU-MK and BFU-MK and with GM-CSF and KL at the level of CFU-MK. Although FL alone exhibited a limited potential in sustaining long-term megakaryocytopoiesis in vitro, it synergistically augmented the ability of IL-3 and KL, alone or in association, to promote long-term megakaryocytopoiesis. These data indicate that multiple cytokines are necessary to optimally stimulate the proliferation of both classes of MK progenitor cells and that FL plays a significant role in this process by amplifying the MK-CSA of GM-CSF, IL-3, and KL.

Shpall, EJ, et al. (1998). "Effect of CD34+ peripheral blood progenitor cell dose on hematopoietic recovery." [39]

The CD34+ cell surface antigen is expressed on progenitor cells required for blood stem cell transplantation. The number of cells expressing CD34+ can be used to assess the peripheral blood progenitor cell (PBPC) graft quality and predict hematopoietic recovery after engraftment. Because there is considerable variability among centers in the determination of CD34+ cell counts, standardizing flow cytometry methodology is essential. It is necessary to define a minimum safety threshold CD34+ cell dose for hematopoietic cell transplantation. This minimum dose would define a cell number in the graft, below which a proportion of patients would be expected to have delayed hematopoietic recovery or failure to engraft. We reviewed data from numerous studies. Although 1-2 x 10(6) CD34+ cells/kg can be considered an adequate graft, available data suggested that doses >5 x 10(6) CD34+ cells/kg were associated with more rapid engraftment and a lower probability of graft failure. The risk of delayed recovery was inversely related to CD34+ cell dose. Delayed recovery may result in greater transfusion requirements, longer hospitalization, increased antibiotic use and growth factor support, and higher health care costs. The extent of prior chemotherapy and radiation treatment are major risk factors for poor PBPC collection. To achieve an optimal CD34+ cell yield, PBPC collection should be initiated early during therapy. PBPC collection should be coordinated with the anticipated number of chemotherapy cycles, duration of chemotherapy, interval between chemotherapy and apheresis, need for radiotherapy, and exposure to the more progenitor cell-toxic drugs such as carmustine or busulfan.

## **10.0 Additional Information**

### **10.1 References**

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### Appendix 3

Component Quality Monitoring Matrix/Lot Release Specifications											
Sample:	Date of Testing:						Sample Start Date:			Sample Harvest Date:	
Test	1	2	3	4	5	6	7	8	Test Lab	Expected/Acceptable	Result
Nucleated Cell Count									BMT, EVE	> 1 x 10 <sup>6</sup> and < 2 x 10 <sup>9</sup>	
Differential									HEM	normal (ANC > 1500/ $\mu$ l)	
Blood Typing									HEM	Same as at recruitment	
Cell Viability									BMT, EVE	$\geq$ 70% prior to transplant	
Flow Cytometry									FLOW	Detectable CD41	
Clonogenic Assay									EVE	Detectable CFU-MK	
Residual TPO									EVE	< 2 ng/ml	
Bacterial Contamination									MICRO	negative	
Fungal Contamination									MICRO	negative	
Gram Stain									MICRO	negative	
Staph Endotoxin									EVE	negative	
Mycoplasma									BIONIQUE	negative	
TH Breast Cancer Detection									TH	negative (Breast Ca Pts Only)	
Malignant Contamination									DMB	< 1 log expansion	
Platelet Function Assay									HEM	normal	
Platelet Aggregation Assay									COAG	normal	
TPO Antibody Assay									MGH	undetectable	
	Culture Step Key	1	2	3	4	5	6	7	8		Test Lab Key
<b>Instructions:</b> Mark at the top of each column the time and date of testing. Check off the boxes in each column when each corresponding test has been successfully performed.											
<b>Important:</b> If an unacceptable result is obtained, notify the study coordinator. Do not release the sample for transplant until negative microbiology and endotoxin test results are obtained.											

BMT = Bone Marrow Transplant  
 EVE = Ex Vivo Expansion  
 HEM = Hematology  
 FLOW = Flow Cytometry  
 MICRO = Microbiology  
 MRL = MRL Reference Laboratory  
 BIONIQUE = BIONIQUE Testing Laboratory  
 TH = Immunohistochemistry  
 DMB = Diagnostic Molecular Biology  
 COAG = Special Coagulation Laboratory  
 MGH = Massachusetts General Hospital